AD_____

Award Number:
W81XWH-07-1-0382

TITLE:

Mechanistic Basis of Calmodulin Mediated Estrogen Receptor Alpha Activation and Antiestrogen Resistance

PRINCIPAL INVESTIGATOR:

Urbauer, Jeffrey L., PhD
Bieber Urbauer, Ramona J., MS
Jolly, Carrie E., PhD

CONTRACTING ORGANIZATION:
University of Georgia Research Foundation Inc.
Athens, GA 30602-7411

REPORT DATE:

June 2010

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2010	Final	15 MAY 2007 - 14 MAY 2010

4. TITLE AND SUBTITLE
Mechanistic Basis of Calmodulin Mediated Estrogen Receptor Alpha Activation and Antiestrogen Resistance

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0382

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Urbauer, Jeffrey L. (Principle Investigator) urbauer@chem.uga.edu
Bieber Urbauer, Ramona J. (Senior Scientist)

Jolly, Carrie E. (Postdoctoral Researcher)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Georgia

Athens, GA 30602-7411

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Estrogen receptor alpha (ERa) is the principle chemotherapeutic target for estrogen dependent breast cancers. Calmodulin (CaM) is an obligatory ERa activator. Moreover, antiestrogens (tamoxifen) bind tightly to CaM, and some therapeutic benefits of antiestrogens for breast cancers are hypothesized to derive from this interaction. The purpose and scope of the research is to define the structural requisites of ERa activation by CaM and the relationship between tamoxifen binding to CaM, CaM oxidation and antiestrogen resistance. We have localized and refined our understanding of the CaM binding sites on ERa. We demonstrated that the high affinity CaM binding region of ERa forms both helical and random coil structure when bound to CaM. We demonstrated that tamoxifen, hydroxytamoxifen and raloxifene binding to CaM are eliminated when the methionine residues of CaM are oxidized. We determined that oxidation of the methionine residues in CaM does not eliminate CaM binding to ERa. The results suggest a mechanism whereby antiestrogen resistance is exacerbated by oxidative stress.

15. SUBJECT TERMS
estrogen receptor alpha, calmodulin, activation, oxidative stress, antiestrogen resistance, tamoxifen, structure, NMR

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION:

Estrogens and the alpha isoform of the estrogen receptor (ERa) are central to estrogen-dependent breast cell carcinoma induction and proliferation. The principal target for systemic endocrine/antiestrogen therapy is ERa, underscoring its biological relevance and medical importance. Despite the apparent wealth of functional and structural information on ERa, the molecular mechanism of ERa activation is sorely incomplete, as only recently has it been established that calcium-dependent activation by calmodulin (CaM) is essential for estrogen-dependent ERa activity, and that the true “active” species is the CaM-ERa complex. This unexpected result has left a glaring gap in our fundamental understanding of ERa activation. CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM). The therapeutic effects of antiestrogens, like those of other CaM antagonists, are indicated to be due, in part, to the direct interaction with CaM. Furthermore, conditions of high oxidative stress and high levels of reactive oxygen species in breast cancer tissues, which are closely linked to antiestrogen resistance, result in oxidation of important methionine residues in CaM, resulting in accumulation of oxidized CaM species and altered function. Finally, CaM is directly implicated in the observed increased tamoxifen resistance associated with unusually high protein kinase A (PKA) levels, as PKA phosphorylates a serine (Ser) residue in the CaM binding domain of ERa, resulting in structural changes in ERa. Because CaM is essential for ERa activation, because of the mounting evidence for the direct involvement of CaM in antiestrogen therapy and antiestrogen resistance, structural and mechanistic details of CaM interactions with ERa and antiestrogens, and the role of posttranslational modifications (CaM oxidation, ERa phosphorylation) on CaM regulation of ERa must be a high priority. Thus, the scope of our research is to define the molecular mechanism, including the structural details, by which CaM activates estradiol-dependent ERa transcription, to demonstrate and define the role of oxidative stress in mediating CaM-ERa and CaM-antiestrogen interactions, and to establish and characterize the role of CaM in PKA-induced antiestrogen resistance

BODY:

General Introductory Statement, Final Report. Our research plan and goals for this project were aggressive and risky. Nevertheless, we were able to make excellent progress on the tasks and subtasks. Although we did not achieve some of our final goals, we made excellent progress toward them and have now also demonstrated the feasibility necessary for the research to continue. Along with some of the significant discoveries we have made, our progress and the feasibility we've demonstrated have positioned the research very well for continued extramural support. Thus, we believe that there will continue to be exceptional returns on the investment of the CDMRP and Breast Cancer Program from our research effort.

Despite the lack of funding we are continuing to pursue the studies proposed, and have in addition initiated new studies to assist in addressing the original goals. These include some structural studies using small angle X-ray scattering (SAXS) and studies to detect and quantify oxidized CaM species in breast tumor cells. These are described below.

Task 1: Define the molecular mechanism, including the structural details, by which CaM activates estradiol-dependent ERa transcription (Months 1-36).

The overall goal here is to describe, from a structural perspective, how CaM binding to ERa activates the receptor. Our principle structural tool is NMR spectroscopy.

<u>*Subtask a).*</u> *Produce ERa and CaM (isotopically labeled and unlabeled) for NMR studies (Months 1-8).* For reference, the domain organization of the estrogen receptor is shown in **Figure 1**. We have produced constructs of the ligand binding domain of ERa (see **Figure 2** and **Figure 3**). The control is the ligand binding domain without the CaM binding region (residues 302-552). The construct with the CaM binding region includes residues 286-552. This construct binds CaM in a Ca^{2+} dependent manner (**Figure 3**) as does full length ERa. We've produced His-tagged construct for immobilization studies (SPR biosensor, Biacore, **Figure 2**). We've produced samples of these proteins that are isotopically labeled for NMR studies (below)(*1, 2*).

We have now succeeded in producing samples of the ligand binding domain both with and without the CaM binding region amenable for NMR studies. This is a major achievement. We now have been able to collect NMR spectra of uniformly isotopically labeled samples of the ligand binding domain of ERa (**Figure 4**). This was a major achievement this year. As described in the previous report, our goal was to produce ERa for our studies with native cysteine residues (i.e. not carboxymethylated, as is nearly universally done for most other studies) in order to most closely mimic the native state. The spectrum shown is a TROSY spectrum on a protein that is <u>*not*</u> deuterated. For an all-helical protein (less chemical shift dispersion) without deuteration, the spectrum is excellent. We now have made perdeuterated samples of the ligand binding domain and

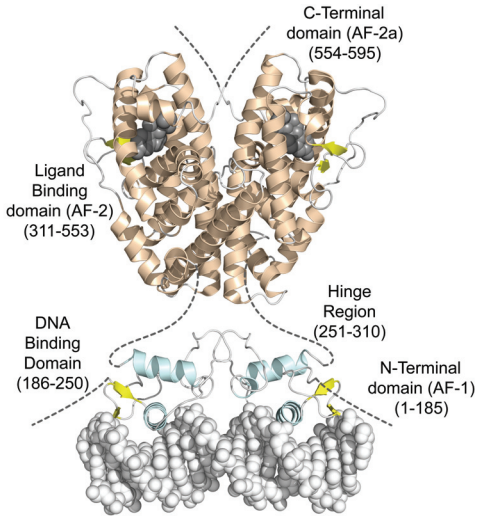


Figure 1: Structural organization of the estrogen receptor dimer. The X-ray crystal structure of the LBD (tan) of ERα with estrogen (gray, space-filling) bound is from Gangloff et al. (*1*) (PDB identifier 1QKU). The X-ray crystal structure of the DBD (blue) of ERα bound to DNA is from Schwabe et al. (*2*) (PDB identifier 1HCQ). No high resolution structural information is available for the AF-1, AF-2a and hinge domains. We have determined the putative CaM binding region is at the C-terminal end of the hinge region (residues 287-311), which includes K302, K303 and S305, all of which can be posttranslationally modified (lysines acetylated, serine phosphorylated).

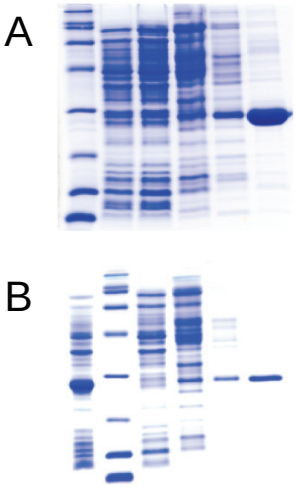


Figure 2. A). Analysis of His_6-ERa(302-552) Purification. Histidine-tagged ERa ligand binding domain, encompassing residues 302-552, was selectively purified from crude bacterial lysate by affinity chromatography on Estradiol Sepharose 6B. Purification of His_6-ERa(302-552) was performed under denaturing conditions. Fractions were collected at each purification step and subjected to SDS-PAGE (15%). Lane 1: molecular weight marker. Lane 2: E_2-Sepharose column flow-through. Lanes 3-5: wash fractions 1 through 3, respectively. Wash buffer 1 contained 1 M urea, and wash buffers 2 and 3 contained 3 M and 5 M urea. Lane 6: elution of His_6-ERa(302-552) with buffer containing 5 M urea and 50 mM E_2. **B)**. Analysis of ERa(286-552) Purification. ERa residues 286-552 was purified under denaturing conditions by affinity chromatography on Estradiol Sepharose 6B. All buffers contained differing concentrations of urea. Fractions were collected at each purification step and subjected to SDS-PAGE (15%). Lane 1: whole cell bacterial lysate. Lane 2: molecular weight marker. Lane 3: E_2-Sepharose wash fraction 1. Lane 4: wash fraction 2. Lane 5: wash fraction 3. Lane 6: pure ERa(286-552). Wash buffers 1-3 contained 1 M, 3 M, and 5 M urea, respectively. The elution buffer contained 5 M urea with 50 mM E_2.

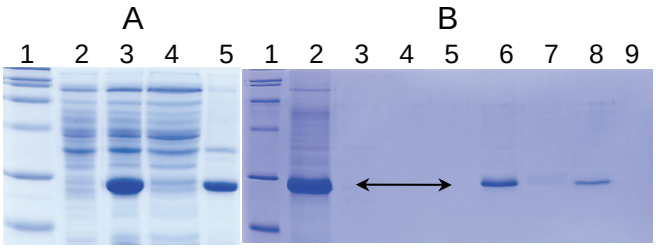


Fig. 3. SDS-PAGE analysis of $ERa_{(286-552)}$ production (A) and binding by CaM-Sepharose (B) (arrow indicates $ERa_{(286-552)}$). A) 1, markers: 2, whole cell extract, uninduced cells: 3, whole cell extract from cells induced with IPTG: 4, supernatant after cell lysis and centrifugation: 5, pellet after cell lysis and centrifugation B) 1, markers: 2, $ERa_{(286-552)}$ after gel filtration chromatography (this protein is loaded onto the CaM-Sepharose): 3, column load flow-through: 4, first wash: 5, second wash: 6, EDTA eluate, first fraction: 7, EDTA eluate, second fraction: 8, CaM-Sepharose resin prior to $ERa_{(286-552)}$ elution: 9, CaM-Sepharose resin after $ERa_{(286-552)}$ elution.

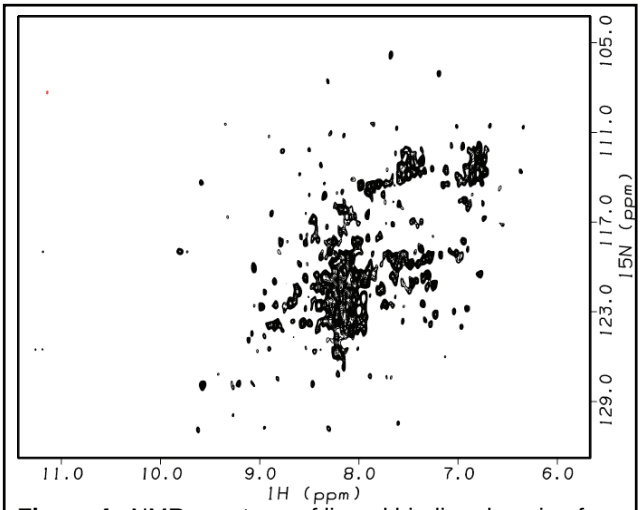


Figure 4. NMR spectrum of ligand binding domain of ERa. Shown is the ^{1}H, ^{15}N-HSQC (TROSY) spectrum of residues 302-552 of the ligand binding domain of ERa ($ERa_{302-552}$). The mass of the dimer (including the His-tag and linker, but without consideration of the ^{15}N) is 61,916.6 Da. The chemical shift dispersion is typical for all helical proteins. The protein is NOT deuterated. The spectrum was acquired at 900 MHz.

the extended ligand binding domain that includes the CaM binding region. We are now well on our way completing our original goals.

<u>*Subtask b).*</u> *Perform NMR experiments on the complex between CaM and ERa with E2 (estrogen) bound and the CaM-ERa complex with TAM bound (Months 8-18).* As discussed above under "*Subtask a*", we have just now been able to acquire NMR spectra of constructs of the ligand binding domain of ERa. We will pursue NMR studies of the complexes with CaM using discretionary funds, and we will apply for additional extramural to continue these important studies.

As described in the previous report, we have also initiated a complementary, but more limited, approach to understanding some of the structural aspects of the interaction between ERa and CaM. In this approach we are defining the structural details of the complex of CaM with the CaM binding region only of ERa. In general, these types of studies of CaM interactions with binding domains from protein targets have been very successful (*3, 4*). Thus, this

approach will enable some of the important structural aspects of this interaction to be resolved. This component is well underway, as indicate in the previous report, and should be completed soon.

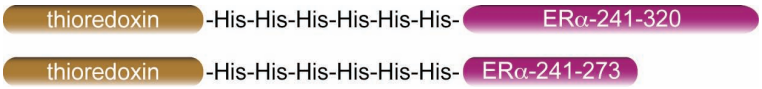


Figure 5. Schematic representations of the proteins Trx-ERA$_{241-320}$ (top) and Trx-ERA$_{241-273}$ (bottom), used in studies to localize the CaM binding region of ERa.

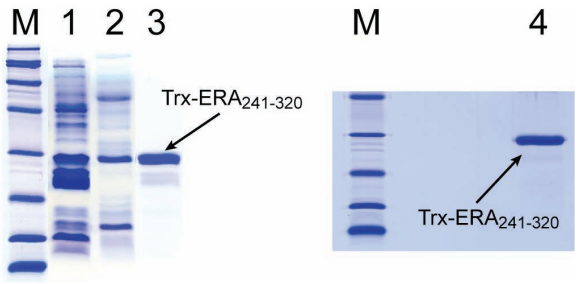


Figure 6: SDS-PAGE assessment of the production and purification of Trx-ERA$_{241-320}$. The gene encoding Trx-ERA$_{241-320}$ was cloned into the pET-32a vector, which was then transformed into *E. coli* BL21(DE3)-RIL for expression. The bacterial were grown on M9 minimal media and induced with IPTG. The cells were lysed, and Trx-ERA$_{241-320}$ was purified by IMAC chromatography, affinity chromatography with immobilized CaM in the presence of Ca^{2+} (elution with EDTA), and HPLC with elution using an acetonitrile gradient. M=molecular weight marker lanes. Lane 1, crude lysate. Lane 2, following IMAC chromatography. Lane 3, following elution from the immobilized CaM column with EDTA. Lane 4, following final purification using HPLC.

First we defined and localized the CaM-binding region of ERa. Our strategy was to examine CaM binding to different segments of the hinge region of ERa (and N-terminal end of the ligand binding domain). In order to do this, we produced two constructs to study their interactions with CaM. The first was a protein construct consisting of the hinge region of ERa and the N-terminal end of the ligand binding domain (residues 241-320) fused to an affinity tag (His_6-tag) and thioredoxin for solubility (**Figure 5**). We call this protein Trx-ERA$_{241-320}$. We also produced a similar protein construct, but with only residues 241-273 of ERa, as a "control" (**Figure 5**). We call this protein Trx-ERA$_{241-273}$. Because CaM binds tightly to the N-terminal extended ligand binding domain of ERa (residues 286-552, see above), we hypothesized that Trx-ERA$_{241-320}$ would bind tightly to CaM, but that Trx-ERA$_{241-273}$ would not.

The genetic constructs encoding these proteins were subcloned into an appropriate expression vector for bacterial expression, and the proteins were produced in *E. coli*. Production and purification of Trx-ERA$_{241-320}$ is shown in **Figure 6**. The His_6 tag enabled purification by immobilized metal affinity chromatography (IMAC). In the case of the longer protein, Trx-ERA$_{241-320}$, we demonstrated tight, Ca^{2+}-dependent binding to CaM, and this was incorporated into the purification protocol. Final purification was by reversed-phase HPLC, resulting in very pure protein for more detailed studies. Purification of Trx-ERA$_{241-273}$ was by IMAC only (**Figure 7**).

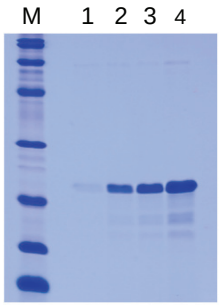


Figure 7: SDS-PAGE of purified Trx-ERA$_{241-273}$. Trx-ERA$_{241-273}$ was produced as described above (**Figure 4**), and purified using IMAC. Lanes 1-4; 1, 5, 10, and 20 μg of purified Trx-ERA$_{241-273}$, respectively

Studies to verify Ca^{2+}-dependent binding of CaM to Trx-ERA$_{241-320}$ are shown in **Figure 8**. In the presense of Ca^{2+}, Trx-ERA$_{241-320}$ binds very tightly to immobilized CaM (CaM-Sepharose 4B), and is not eluted even at very high ionic strength (1 M NaCl). Only when the Ca^{2+} is chelated with EDTA, does Trx-ERA$_{241-320}$ elute from the resin (lane 8 of panel 'A'), demonstrating a tight, Ca^{2+} dependent, specific interaction of Trx-ERA$_{241-320}$ with CaM. In the absence of Ca^{2+} (panel 'B'), some nonspecific, ionic strength dependent binding is observed, with high ionic strength removing all Trx-ERA$_{241-320}$ from the resin (nearly all Trx-ERA$_{241-320}$, is removed by 100 and 200 mM NaCl, lanes 4 and 5). Controls were performed, both in the

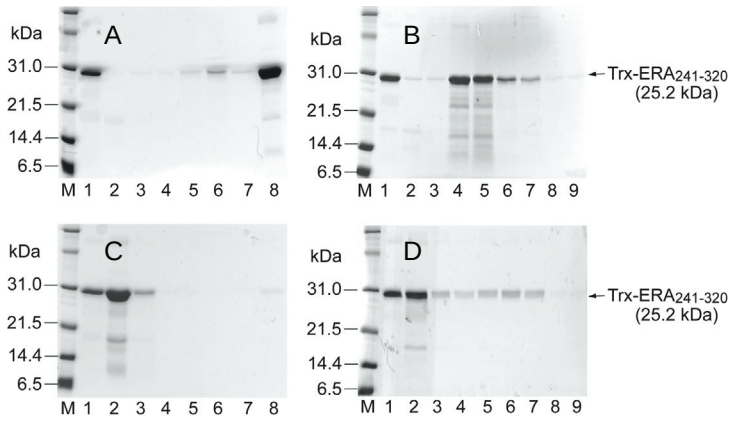


Figure 8: Trx-ERA$_{241-320}$ binds to Ca^{2+}-CaM. SDS-PAGE was used to analyze eluents from immobilized CaM resin (or control resin without immobilized CaM) for affinity of Trx-ERA$_{241-320}$ for the resins either in the presence or absence of Ca^{2+}. (A) In the presence of Ca^{2+}, Trx-ERA$_{241-320}$ binds tightly to immobilized CaM (CaM-Sepharose 4B), is not released even by high ionic strength, and is eluted selectively by addition of a Ca^{2+} chelator (EDTA). (B) When no Ca^{2+} is present, there is some affinity of Trx-ERA$_{241-320}$ for immobilized CaM, but Trx-ERA$_{241-320}$ is eluted from the immobilized CaM by high ionic strength. (C)/(D) These are controls for 'A' and 'B', where resin (Sepharose 4B) alone, without immobilized CaM, is used. For 'A' and 'C', the equilibration buffer for the affinity resin and the buffer solution for the CaM is 20 mM Tris-HCl, pH 7.5, with 2 mM Ca^{2+}, and the elution buffer is 20 mM Tris-HCl, pH 7.5, with 2 mM EGTA and 1 mM $NaCl_2$. For 'B' and 'D', the equilibration buffer (and the buffer solution for the CaM is 20 mM Tris-HCl, pH 7.5, with 1 mM EDTA, and the "elution" buffer is 20 mM Tris-HCl, pH 7.5 with 1 M NaCl and 10 mM $CaCl_2$. M=molecular weight markers. Lane 1, purified Trx-ERA$_{241-320}$ protein in equilibration buffer. Lane 2, load flow-through. Lane 3, equilibration buffer. Lanes 4-7, equilibration buffer with 100, 200, 500, or 1000 mM NaCl, respectively. For 'A' and 'C', lane 8 is elution buffer. For 'B' and 'D', lane 8 is equilibration buffer, and lane 9 is elution buffer.

presence of Ca^{2+} and its absence, using Sepharose 4B without immobilized CaM (panels 'C' and 'D'). No significant interactions of Trx-ERA$_{241-320}$ with the resin alone were observed.

The results in **Figure 8** indicate a tight, Ca^{2+}-dependent interaction of Trx-ERA$_{241-320}$ with CaM. Based on these results, and the results above demonstrating that CaM binds tightly to the N-terminal extended ligand binding domain of ERa (residues 286-552, see above), we hypothesized that the high affinity binding site of ERa for CaM lies in the C-terminal half of the hinge region. To test whether the N-terminal part of the hinge region would bind tightly to CaM, we tested for binding of Trx-ERA$_{241-273}$ to CaM. These results are shown in **Figure 9**. These experiments were performed precisely as those with Trx-ERA$_{241-320}$. A weak, ionic strength dependent interaction of Trx-ERA$_{241-273}$ is observed, both in the presence and absence of Ca^{2+} (panels 'A' and 'B'). No interaction with the Sepharose 4B resin (without immobilized CaM) is observed (panels 'C' and 'D'). These results suggested the possibility of a weak interaction of CaM with this region of ERa.

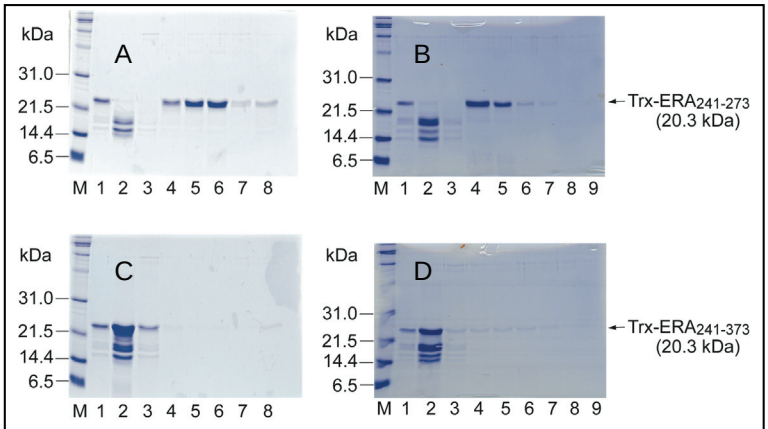


Figure 9: Trx-ERA$_{241-273}$ does not bind specifically to CaM. SDS-PAGE was used to analyze eluents from immobilized CaM resin (or control resin without immobilized CaM) for affinity of Trx-ERA$_{241-273}$ for the resins either in the presence or absence of Ca^{2+}. These experiments were performed in a manner identical to those in **Figure 6**. The panels here also correspond to those in **Figure 6**.

In order to address the possible weak interaction of residues 241-273 of ERa with CaM, we produced two additional control Trx constructs, one called Trx-RNDM consisting of Trx-ERA$_{241-273}$ where the amino acid sequence of the region 241-273 was randomized. The other control construct was Trx alone (including the His_6 region). These were produced and purified (**Figure 10**) in a manner very similar to the initial constructs (see previous year report). The results of binding to CaM are shown in **Figure 11** and **Figure 12**.

The results in **Figure 11** and **Figure 12** indicate a non-sequence specific interaction between residues 241-273 of ERa and CaM, and a very low affinity of Trx with CaM. Thus, the weak interaction between residues 241-273 and CaM is simply a non-specific interaction. The

affinity between ERa and CaM is, therefore, due to the interaction of CaM and the region of ERa comprising residues 287-311.

Cam binds basic, amphiphilic regions, and based on our results (above) showing that CaM binds with high affinity to an ERa ligand binding domain located between residues 274 and 320 (see above), and we hypothesized the existence of a high-affinity CaM binding domain comprised of

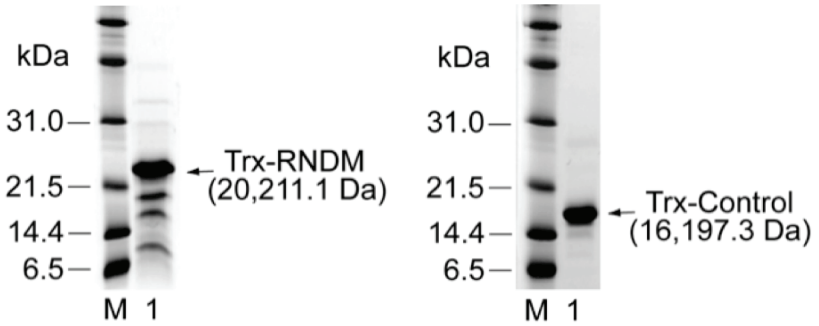


Figure 10: (**Left**). SDS-PAGE assessment of the production/purification of Trx-RNDM (Trx-$ERA_{241-273}$, where the amino acid sequence 241-273 was randomized). The protein was produced as described previously for our other Trx constructs and purified by IMAC chromatography. The indicated mass is the exact mass. (**Right**). SDS-PAGE assessment of the production/purification of Trx-Control (Trx only). The protein was produced as described previously for our other Trx constructs and purified by IMAC chromatography. The indicated mass is the exact mass.

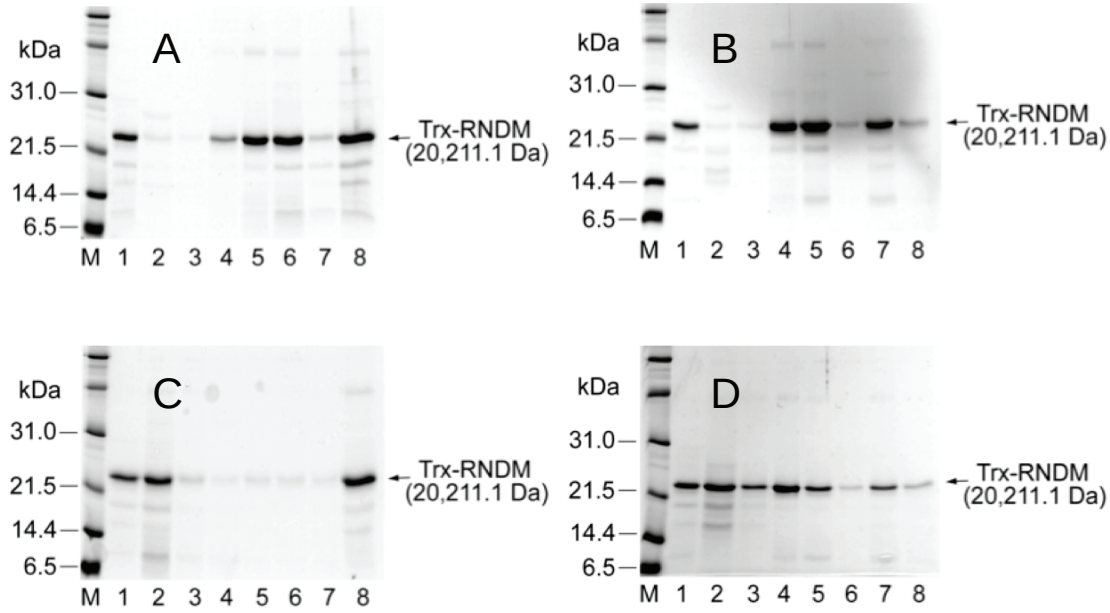


Figure 11: Trx-RNDM (Trx-$ERA_{241-273}$, where the amino acid sequence 241-273 was randomized) binds weakly, nonspecifically to CaM. SDS-PAGE was used to analyze eluents from immobilized CaM resin (or control resin without immobilized CaM) for affinity of Trx-RNDM for the resins either in the presence or absence of Ca^{2+}. (A) In the presence of Ca^{2+}, Trx-RNDM binds apparently to immobilized CaM (CaM-Sepharose 4B), is mostly released by high ionic strength, with some remaining protein eluted by addition of a Ca^{2+} chelator (EDTA). (B) When no Ca^{2+} is present, there is still some apparent affinity of Trx-RNDM for immobilized CaM, but most is eluted from the immobilized CaM by high ionic strength. (C)/(D) These are controls for 'A' and 'B', where resin (Sepharose 4B) alone, without immobilized CaM, is used. The results in C particularly indicate non-specific interaction of this construct with the resin, and not with the calmodulin.For 'A' and 'C', the equilibration buffer for the affinity resin and the buffer solution for the CaM is 20 mM Tris-HCl, pH 7.5, with 2 mM Ca^{2+}, and the elution buffer is 20 mM Tris-HCl, pH 7.5, with 2 mM EGTA and 1 mM $NaCl_2$. For 'B' and 'D', the equilibation buffer (and the buffer solution for the CaM is 20 mM Tris-HCl, pH 7.5, with 1 mM EDTA, and the "elution" buffer is 20 mM Tris-HCl, pH 7.5 with 1 M NaCl and 10 mM $CaCl_2$. M=molecular weight markers. Lane 1, purified Trx-$ERA_{241-320}$ protein in equilibration buffer. Lane 2, load flow-through. Lane 3, equilibration buffer. Lanes 4-7, equilibration buffer with 100, 200, 500, or 1000 mM NaCl, respectively. Lane 8 is elution buffer.

residues 287-311 of ERa (**Figure 13**). We therefore performed experiments to verify if this peptide would bind with measurable affinity to CaM. We used a synthetic peptide corresponding to residues 287-311 of ERa, which we call $HERA_{287\text{-}311}$. For controls, we used a

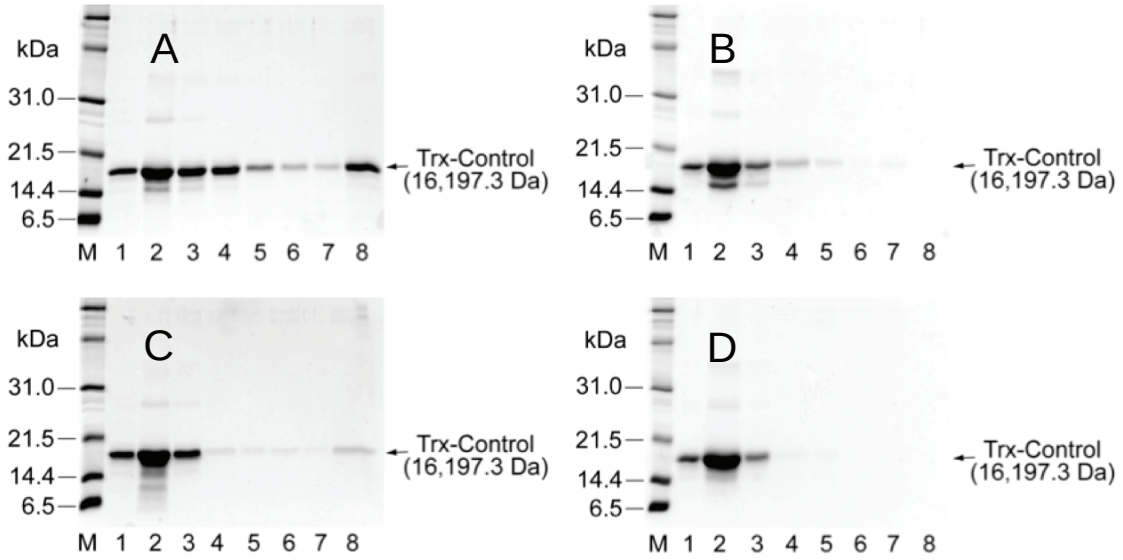


Figure 12: Trx does not bind specifically to CaM. SDS-PAGE was used to analyze eluents from immobilized CaM resin (or control resin without immobilized CaM) for affinity of Trx for the resins either in the presence or absence of Ca^{2+}. These experiments were performed in a manner identical to those in **Figure 5**. The panels here also correspond to those in **Figure 5**. There is a modest affinity of Trx for CaM in the presence of Ca^{2+} (A), but it is abrogated by high ionic strength

peptide corresponding to residues 295-311 of ERa ($HERA_{295\text{-}311}$) and a peptide known to bind to CaM with very high affinity (CaM binding domain of the myosin light chain kinase, MLCK, dissociation constant in the nM regime. These sequences are shown in **Figure 13**.

We used electrophoretic mobility shift assays (EMSA) to determine the relative abilities of these peptides to form complexes with CaM in non-denaturing polyacrylamide gels (**Figure 14**). The MLCK peptide binds very tightly to the CaM. The complex is clearly visible in the gel, little free CaM remains at a 1:1 ratio of peptide:CaM, and no free CaM is observed at higher ratios. Likewise, a complex between $HERA_{287\text{-}311}$ and CaM is observed even at low peptide:CaM ratios. The results indicate that the affinity of this complex is not as high as the MLCK:CaM complex. Finally we do not detect complex formation between the shorter $HERA_{295\text{-}311}$ peptide and CaM. This indicates that residues 287-294 contribute substantially to the affinity of ERa for CaM. This is important, because recent studies (*5, 6*) suggest that these residues, 295-311 of ERa, are the CaM binding domain, but this is clearly not correct or complete.

MLCK	**ARRKWQKTGHAVRAIGRLSS**
$HERA_{287\text{-}311}$	**287-RAANLWPSPLMIKRSKKNSLALSLT-311**
$HERA_{295\text{-}311}$	**PLMIKRSKKNSLALSLT**

Figure 13: Amino acid sequences of peptides used in these studies. Shown are sequences corresponding to residues 287-311 of the human estrogen receptor alpha ($HERA_{287\text{-}311}$), residues 295-311 ($HERA_{295\text{-}311}$), and the high affinity CaM binding sequence of myosin light chain kinase (MLCK), which is used as a control. Blue, positively charged. Orange, hydrophobic.

We have performed fluorescence titration studies to characterize the binding of $HERA_{287\text{-}311}$ to CaM. Because the amino acid sequence of $HERA_{287\text{-}311}$ includes a tryptophan residue, and because there are no tryptophan residues in CaM, we can use fluorescence spectroscopy,

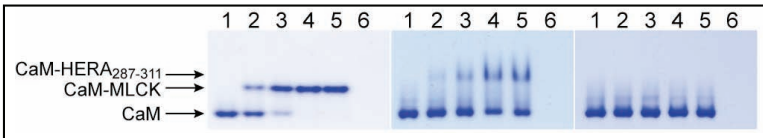


Figure 14: Affinity of CaM for $HERA_{287-311}$ and $HERA_{292-311}$. Electrophoretic mobility shift assays (EMSA) were performed to assess qualitatively the relative affinities of CaM for $HERA_{287-311}$ (*center*) and $HERA_{295-311}$ (*right*). A control experiment (*left*) with the CaM binding region of myosin light chain kinase (MLCK), which binds to CaM with high affinity (nM), was also performed. Solutions of constant CaM concentration and increasing peptide concentrations were subjected to electrophoresis using non-denaturing PAGE. Lane 1, 25 μM CaM only. Lane 2-5, 25 μM CaM plus 12.5, 25, 50, or 100 μM peptide. Lane 6, 100 μM peptide only. For $HERA_{295-311}$, no complex formation or decrease in free CaM with increasing peptide concentration could be detected, indicating that the affinity of this peptide for CaM is significantly lower than the affinity of $HERA_{287-311}$ for CaM. *Experimental details* - The solutions included 2.5 mM MOPS, 5.0 mM $CaCl_2$, 5.0 mM KCl, pH 7.0. The solutions were mixed and incubated at room temperature for 10 minutes prior to electrophoresis. A 15% non-denaturing gel (with a stacking gel) was used, and electrophoresis was performed at 150 volts at room temperature for 1 hour and 40 minutes. Proteins were visualized with Coomassie R-250 staining.

and intrinsic tryptophan fluorescence, to monitor the interaction of $HERA_{287-311}$ and CaM. Likewise, the MLCK peptide sequence includes a single tryptophan residue, so, as a control, we can monitor binding of this peptide to CaM using fluorescence techniques also. In **Figure 15** are shown fluorescence emission scans of $HERA_{287-311}$ and the MLCK peptide as a function of added CaM. In both cases, the emission wavelength maxima shift to lower wavelengths when the peptides bind to CaM, and the emission intensity increases. These results signify that, in both cases, peptide binding to CaM results in the tryptophan becoming buried in a hydrophobic environment. For $HERA_{287-311}$, the intensity increase is particularly dramatic, indicating that the tryptophan residue is deeply buried in a hydrophobic environment (one of the hydrophobic clefts of the globular domains of CaM). These results indicate clearly that residues 287-294 (including the tryptophan residue at position 292) contribute to the interaction of ERa with CaM.

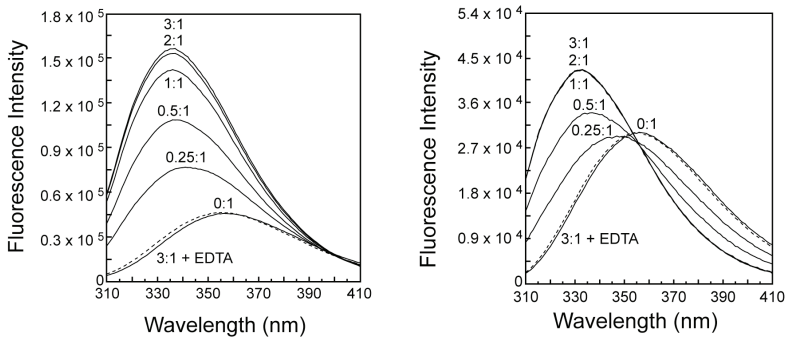


Figure 15: Fluorescence emission spectra of $HERA_{287-311}$ (left) and the MLCK peptide (right) free in solution and bound to Ca^{2+}_4-CaM. The spectra were acquired at various ratios of CaM:peptide (0:1 = no CaM, 3:1 = three fold molar excess CaM over peptide, etc.). The spectra are corrected by subtraction for the small background fluorescence of the added CaM. The excitation frequency was 295 nm. The spectra were acquired in a solutions consisting of 10 μM peptide, 0-30 μM CaM, 50 mM MOPS, 2 mM $CaCl_2$, 100 mM KCl, pH 7.0. The temperature was 25 °C.

The stoichiometry of the interaction initially was unclear. Interestingly, inspection of the results in **Figure 15** indicates that the increase in intensity observed when the $HERA_{287-311}$:CaM ratio is only 0.5:1 is much higher than expected. This suggests that more than one $HERA_{287-311}$ peptide

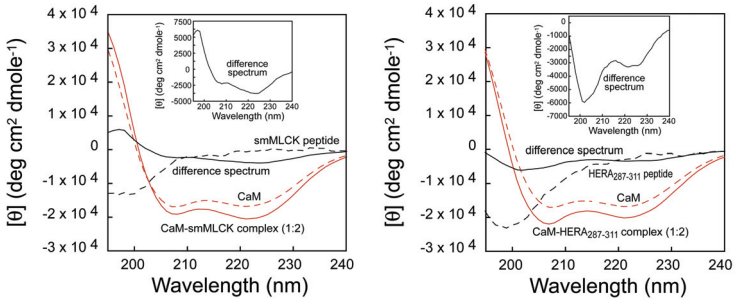


Figure 16. Circular dichroism spectra of CaM, CaM binding peptides, and complexes of CaM with these peptides. The control, CaM binding to the smMLCK peptide indicates considerable helical structure adopted by the peptide when bound (difference spectra). With HERA, the peptide adopts both helical and extended random coil structure when bound to CaM.

is binding to CaM, which suggests that the stoichiometry of the $HERA_{287-311}$:CaM complex is 2:1 rather than 1:1. As it turns out, when the peptide is in large excess (as it is during initial stages of the titration, as we performed them), it appears that CaM is able to bind simultaneously to two of the peptides. However, as the concentration of CaM increases, the entropic imperative forces a 1:1 stoichiometry, as is the usual case of CaM binding to such peptides.

We've also performed circular dichroism studies to determine the secondary structure of the CaM binding region of ERa bound to CaM. In **Figure 16** are shown the results. The control

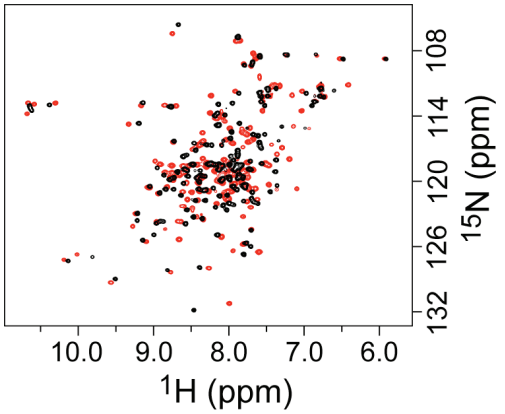


Figure 17: Structural changes in CaM accompanying $HERA_{287-311}$ binding. ^{1}H, ^{15}N-HSQC spectra of uniformly ^{13}C, ^{15}N-labeled $Ca^{2+}{}_4$-CaM, both free in solution (black contours) and bound to $HERA_{287-311}$ (red contours) are shown. The concentrations of CaM and $HERA_{287-311}$ are 0.5 and 1.5 mM, respectively, and the buffer solution is 5 mM d_4-imidazole, 10 mM KCl, 10 mM $CaCl_2$, pH 6.5, with 5% D_2O for an instrumental lock. The spectra were acquired at 25 ℃ and 600 MHz (^{1}H). The spectrum of the complex indicates it to be structurally homogeneous and amenable to continued study by NMR. The chemical shift changes throughout CaM resulting from $HERA_{287-311}$ binding indicate structural changes throughout CaM

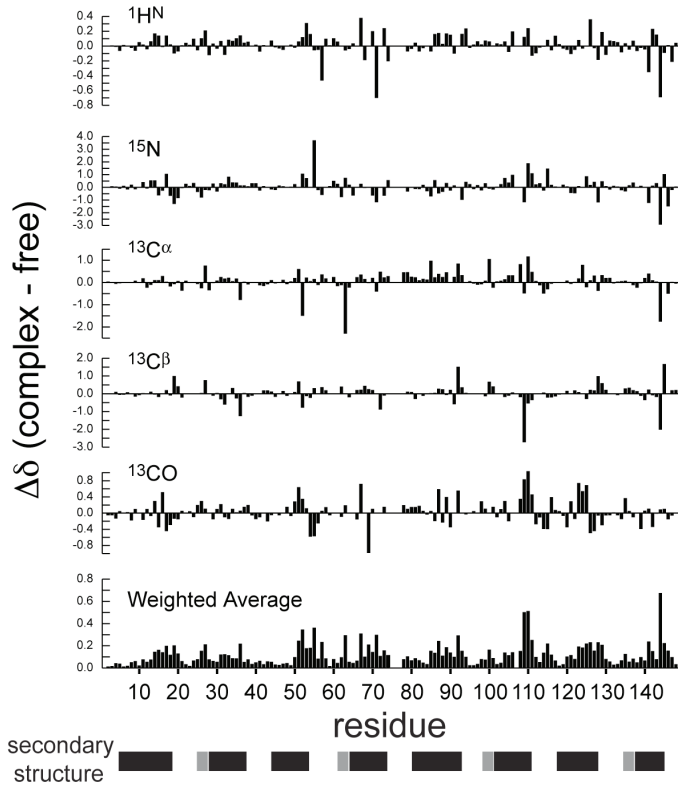


Figure 18: Main chain chemical shift changes in $Ca^{2+}{}_4$-CaM accompanying binding to $HERA_{287-311}$. The changes in the chemical shifts of the main chain and $^{13}C^{\alpha}$ atoms for $Ca^{2+}{}_4$-CaM, which result from binding to $HERA_{287-311}$, are shown as a function of residue. The plot at the bottom shows an absolute value weighted average (**REF**) calculated by normalizing the absolute value of each shift change by the absolute value of the largest change for that nucleus and then averaging for each position. The secondary structure is also shown (dark bars represent alpha helices and light bars beta strands), determined using chemical shift index (CSI) analysis (**REF**).

MLCK peptide adopts nearly complete helical structure when bound to Cam. However, $HERA_{287-311}$ adopts both helical and random coil structure when bound to CaM. This suggests perhaps that the canonical structure of CaM bound to tight binding amphiphilic regions is not represented by the CaM-HERA structure.

We are well on our way to completing a high resolution structure of the complex of $HERA_{287-311}$ and CaM ($Ca^{2+}{}_4$-CaM) using NMR. We have titrated ^{13}C, ^{15}N-labeled CaM with $HERA_{287-311}$ to produce a complex amenable for study. A comparison of the 2D ^{1}H, ^{15}N-HSQC NMR spectra of free CaM and CaM bound to $HERA_{287-311}$, which reflect the structural differences of free CaM and CaM bound to $HERA_{287-311}$, are shown in **Figure 17**. Because the signals in the spectrum of free CaM are assigned, it is clear that structural elements from all parts of the molecule are changed when $HERA_{287-311}$ binds. Moreover, the spectrum of the

complex indicates that the complex is homogeneous in solution, and that determining a high resolution structure using NMR will not be problematic.

Using standard triple resonance assignment methods (HNCA, HN(CO)CA, HNCACB, CB(CACO)NH, etc.) we have assigned the main chain nuclei (and $^{13}C_{\beta}$) of Ca^{2+}_4-CaM bound to $HERA_{287\text{-}311}$. This has allowed us to calculate the chemical shift changes of the nuclei of CaM that accompany $HERA_{287\text{-}311}$ binding, and thus to begin the process of understanding the structural changes that take place in CaM upon $HERA_{287\text{-}311}$ binding. For the assigned nuclei, we have calculated the chemical shift changes, and have also calculated a weighted average over all assigned nuclei (**Figure 18**). Overall, the chemical shift changes are somewhat smaller than those observed when CaM binds to prototypical, canonical tight binding sequences, such as the MLCK peptide (*7*). In the case of $HERA_{287\text{-}311}$ binding, the chemical shift changes suggest that the collapse of CaM around the peptide(s) is not as dramatic as that observed, for instance, for the MLCK peptide. This suggests that the complex is perhaps somewhat elongated relative to, for instance, the complex with the MLCK peptide. We also note some unusual, very large changes in the C-terminal domain (M109 in helix VI, M144 in helix VIII) signifying important structural changes in the C-terminal binding pocket.

We have just recently initiated the use of small angle X-ray scattering (SAXS) methods for our structural studies. We are using SAXS in two ways for this project. First, currently we are using SAXS to assist with determining the NMR-based solution structure of the complex of CaM with a peptide corresponding to the CaM binding region of ERa (residues 287-311). We are using SAXS to assist in defining the relative orientation and spacing of the opposing globular domains of CaM when bound to the ERa peptide, and the overall "envelope" of the structure of the complex (**Figure 19**). This shape information is complementary to the structural information from NMR and can be used in conjunction with the NMR restraint information for refining the final structures.

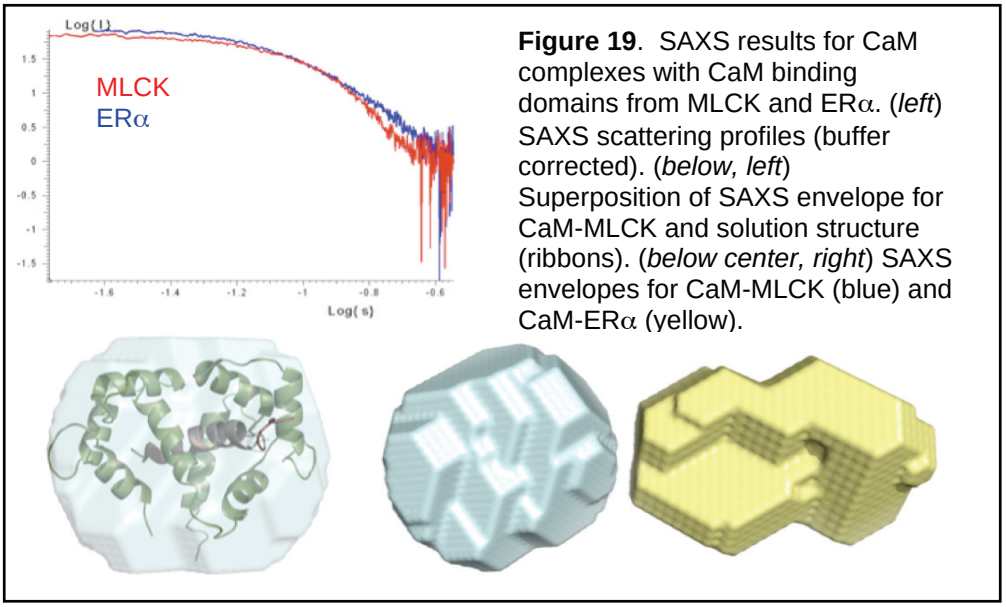


Figure 19. SAXS results for CaM complexes with CaM binding domains from MLCK and ERα. (*left*) SAXS scattering profiles (buffer corrected). (*below, left*) Superposition of SAXS envelope for CaM-MLCK and solution structure (ribbons). (*below center, right*) SAXS envelopes for CaM-MLCK (blue) and CaM-ERα (yellow).

As noted above, from the chemical shift changes it appears that the CaM complex with the HERA peptide is somewhat elongated compared to more canonical complexes. This conjecture is clearly borne out by SAXS data we have collected recently on the complexes of CaM with the ERa peptide, and, as a control, the complex of CaM with a peptide corresponding to the CaM binding region of the smooth muscle MLCK (**Figure 19**). The data were acquired with a Bruker NANOSTAR instrument at Bruker AXS (Madison, WI), were processed and analyzed with the ATSAS (version 2.3.1) suite (*8-10*) and envelopes calculated with DAMMIF (*11*). The samples (1:1 complexes of CaM and peptide) were 600 micromolar, and data

collection was for approximately 1 hour. As shown in **Figure 19**, the envelope calculated with SAXS for the CaM-MLCK peptide complex superimposes very well with the structure determined by NMR (PDB ID 2BBM). The radius of gyration from SAXS and that calculated from the high resolution solution structure are identical (17.0 Å). Moreover, the SAXS envelope calculated for the CaM-ERa complex clearly is elongated relative to the control complex, as expected. We are currently completing the necessary NMR studies before calculating the final structures, and will use these SAXS data in the final refinements to assist with domain orientation as described above.

Our second use for SAXS in this project is for defining structural changes in the ligand binding domain of ERa and the full length ERa when calmodulin binds. We are pursuing NMR based structural studies of the former, but not the latter (due to its size). Similar applications of SAXS to related nuclear hormone receptors have been quite successful (*12*). High resolution (X-ray crystal) structures of the ligand binding domain of ERa and the DNA binding domain are available, and, given these models and our knowledge of the CaM binding domain, we anticipate that structural changes accompanying CaM binding can readily be resolved. We suspect there will be functionally significant changes in the relative orientation of the ligand binding domain and DNA binding domain of ERa when CaM binds, as part of the CaM binding domain is in the intervening region ("hinge" region). Because these complexes are unlikely to crystallize, this approach would be an exceptional alternative to ultimately determine the fundamental structural origins of the activation process. Determining reasonable models of these complexes and the structural changes accompanying CaM binding would be a tremendous accomplishment, and will most likely change the fundamental nature of how the activation process is currently viewed.

Finally, using fluorescence methods *in vivo*, a recent study has shown that posttranslational modifications (phosphorylation) in the CaM binding region (serine 305) by PKA cause substantial, yet unspecified, conformational changes in the ERa leading to tamoxifen resistance (*13*). We consider it likely that the changes seen were the result of altering the ability of CaM to bind. Therefore, we should be able to repeat these experiments under controlled conditions *in vitro* using SAXS measurements of native and modified ERa in the presence and absence of CaM. Measurements of CaM binding affinity to the intact receptor when modified, along with the SAXS results, would then discriminate between an unknown mechanism or one that simply involves changes in CaM affinity for the receptor when modified. Again, this would be a critical discovery, and could lead to fundamental breakthroughs in how antiestrogen resistance develops in breast tumors.

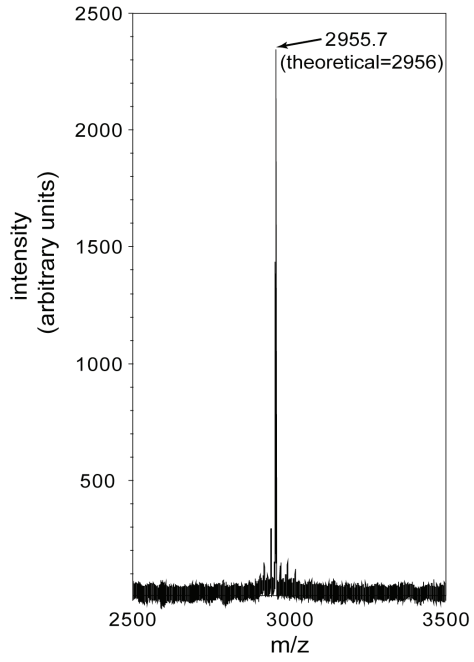


Figure 20. MALDI mass spectrum of uniformly ^{13}C, ^{15}N-labeled $HERA_{287\text{-}311}$. The fusion protein consisted of thioredoxin, a linker with enterokinase cut site and residues 287-311 of HERA (the CaM binding region). Isotopically labeled peptide was produced and purified, with HPLC as the final purification step. The spectrum shown is of the final, purified peptide. The mass is exact.

Finally, the task of determining a high resolution structure of the complex of CaM with the HERA peptide (residues 287-311) is much simplified if uniformly isotopically labeled peptide is available. We have now succeeded in producing recombinant peptide and have isotopically labeled it (**Figure 20**). Producing peptides recombinantly can be

very problematic, and we had some unsuccessful attempts. In one case, in fact, we were able to produce the peptide but it was modified by the cell.

Thus, a significant accomplishment during the last year was the recombinant production of the CaM binding region of ERa, isotopically labeled (**Figure 20**). We used a thioredoxin fusion and enterokinase cut site to produce this peptide. Now that we have produced this peptide, we will be able to easily complete the structure of the CaM-peptide complex. This will be critical to establishing the structural prerequisites for CaM activation of ERa.

<u>Subtask c).</u> *Calculate structures of the complexes using standard NMR computational methods and software (Months 12-36).* We now have all of the components in place to complete the NMR experiments to determine the structures that were goals of this work. We knew up front that these were not simple goals, and that there was a lot of risk associated with them. However, we are very pleased in that we have been able to make the progress necessary to establish clearly the feasibility and we have made good progress towards actually determining the structures. In addition, we are continuing to collect and use SAXS data for determining these structures, and this is expected both to hasten the process and produce higher quality structures. We will continue to seek extramural funding to continue these studies, but, clearly, the CDMRP funds have been critical in getting these studies firmly established.

Task 2: Demonstrate and define the role of oxidative stress in mediating CaM-ERa and CaM-antiestrogen interactions (Months 6-36).

The overall goal here is to determine if oxidative stress and oxidation of CaM resulting from oxidative stress can mediate the complex between CaM and ERa and antiestrogen resistance.

<u>Subtask a).</u> *Produce ERa and wildtype and mutant CaM proteins (isotopically labeled and unlabeled) for NMR, gel shift, and SPR studies (Months 6-18).* This is completed.

Subtask b). Perform NMR experiments on the complex between CaM and TAM (Months 6-24). Recent results have shown that TAM metabolites, rather than TAM itself, are the therapeutically active compounds responsible for the therapeutic benefits of TAM therapy (*14-16*). We have demonstrated that oxidative stress, if methionine oxidation in CaM results, can cause CaM to lose its ability to bind TAM. Our hypothesis is that CaM is no longer inhibited (CaM inhibition contributing to ERa deactivation), and can once again activate TAM (a mechanism for antiestrogen resistance brought about by oxidative stress). We continued these studies to include the effect of CaM oxidation on CaM binding to hydroxy tamoxifen (OHTAM), raloxifene (a different class of antiestrogens), and endoxifen (OHTAM and endoxifen are, putatively, the active metabolites of TAM). As shown in **Figure 21**, oxidation of CaM eliminates the interaction with OHTAM. This is the same result observed with TAM that we reported earlier. The control protein, CaM with all methionine residues substituted by leucine, binds OHTAM as well as wild type CaM. We also demonstrate in **Figure 21** that raloxifene binds to CaM (the first demonstration of this, to our knowledge), and that oxidation eliminates CaM binding to raloxifene also (there is, apparently, a very, very weak interaction of CaMox with raloxifene, most likely a non-specific interaction). These results are important and lend additional credence to our hypothesis concerning CaM oxidation and antiestrogen resistance.

We also plan to perform these types of studies with endoxifen. There is only a single commercial source for endoxifen, and we placed an order for this compound long ago. We just recently received this compound, and will begin these studies shortly.

Finally, we originally had proposed to determine a structure of CaM with TAM bound. Based on the results shown in **Figure 21**, the complex of CaM with OHTAM is much more

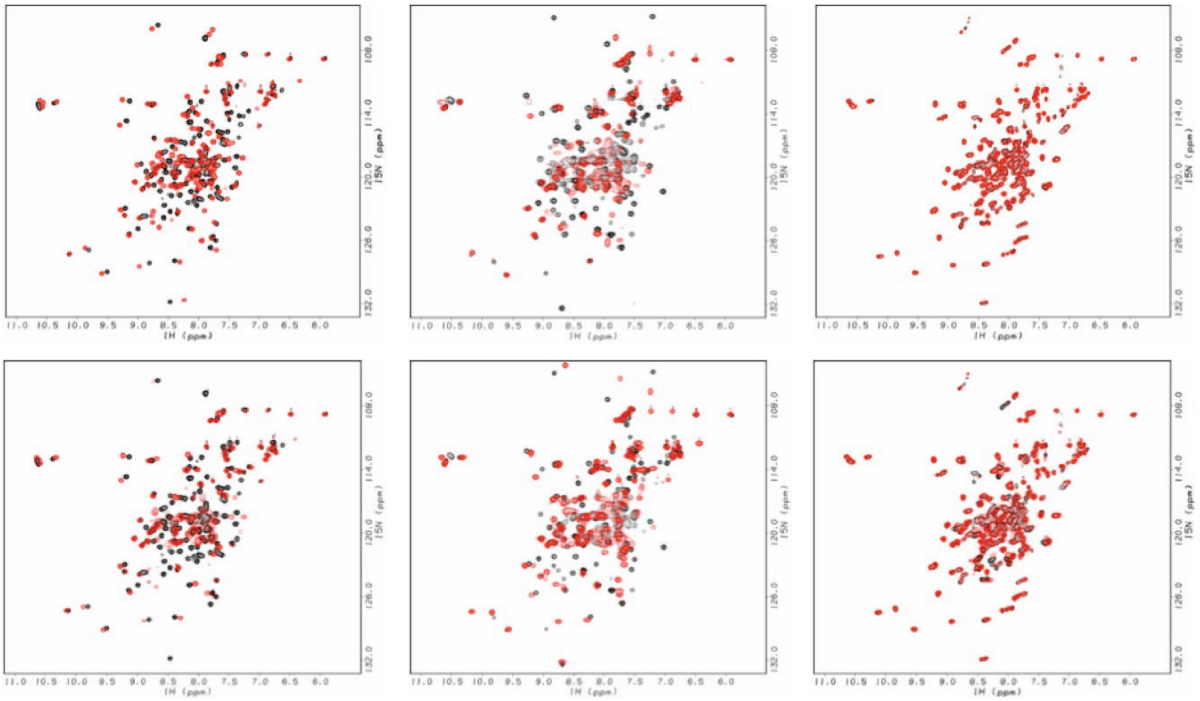


Fig. 21 ^{1}H, ^{15}N-HSQC spectra of ^{15}N, ^{13}C-labeled CaM species. A) Black, CaM. Red, CaM-OHTAM complex. B) Black, L9-CaM. Red, L9-CaM-OHTAM complex. C) Black, CaMox. Red, CaMox plus OHTAM. D) Black, CaM. Red, CaM-raloxifene complex. E) Black, L9-CaM. Red, L9-CaM-raloxifene complex. F) Black, CaMox. Red, CaMox plus raloxifene. The concentration of CaM or CaM species was ~0.5 mM, in 10 mM $CaCl_2$, 10 mM KCl, 5 mM d_4-imidazole, 0.02% sodium azide, 8% D_2O, pH 6.5.

amenable to NMR than is the CaM-TAM structure. This appears to be due to the fact that OHTAM is slightly more soluble than TAM, and we can better saturate CaM with OHTAM in aqueous solution. Given that recent studies suggest endoxifen even more potent therapeutically, once we perform these experiments with endoxifen, we will choose to determine the high resolution structure of either the CaM-OHTAM or the CaM-endoxifen complex, depending on which gives the best spectra and is the best behaved in solution for NMR experiments.

Although we planned during the past year to perform these studies also with endoxifen (a further metabolite of TAM shown recently to be important in ERa inhibition), we did not do so, electing to focus on the NMR structural studies described above. However, we have purchased the endoxifen (only one commercial supplier), and will perform these studies shortly. We will also determine a structure of CaM with one of the TAM metabolites (OHTAM, endoxifen) bound.

Subtasks c-f). These subtasks are in various stages of completion. For instance, we had proposed to use gel mobility assays to determine the effects of CaM oxidation on its ability to bind ERa, but have instead focused on using fluorescence spectroscopy. Most of these subtasks will be included in proposals for future funding, using the data generated to date.

Additional studies addressing this task: Accumulation of oxidized CaM in breast tumor cells). Our hypothesis concerning CaM oxidation and antiestrogen resistance suggests that oxidized CaM species will accumulate in estrogen dependent breast tumor cells, particular cells from old tumors and cells from tumors that have already progressed to antiestrogen resistance status. We have initiated studies to detect and quantify these oxidized species. Initial studies have focused on using monoclonal antibodies to CaM (that cross react well with oxidized CaM species) to detect oxidized CaM species in cell extracts. As increasing numbers of methionine

residues in CaM become oxidized, the mobility decreases on SDS page gels (as we have shown previously (*17*). These studies have just been initiated. To date, we have detected oxidized CaM in common cultured breast cancer cell lines and have just begun studies of actual breast tumor samples. Initial results on early stage tumors (controls, expected to not have any CaM oxidation) indicate no oxidation, as expected. Studies on more advanced tumors are now being performed.

Task 3: Test and describe the direct link between PKA induced antiestrogen resistance and CaM binding to ERa (Months 12-36).

The goal of this task is to test the hypothesis that posttranslational modifications in the CaM binding region of ERa mediate the interaction, and how this might contribute to antiestrogen resistance.

Subtask a). Obtain synthetic peptides corresponding to the CaM binding domain of ERa and phosphorylate at Ser 305 (Months 12-15). This is complete.

Subtask b). Produce ERa and CaM (isotopically labeled and unlabeled) for NMR and SPR experiments (Months 12-15). This is complete.

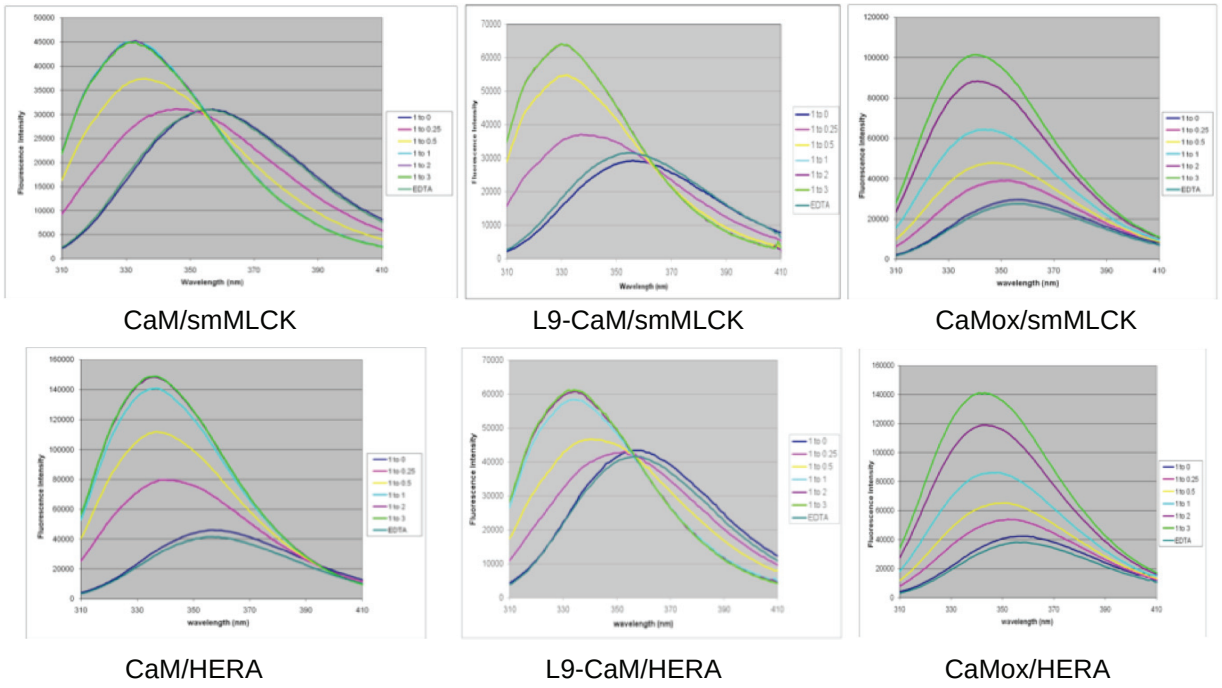


Fig. 22. Titration of HERA (the CaM binding domain of human estrogen receptor alpha, residues 287-311) and smMLCK (the CaM binding domain of the smooth muscle myosin light chain kinase) with CaM, L9-CaM (CaM with all methionine residues replaced with leucines) and CaMox (CaM with all methionine residues oxidized to the sulfoxides). The fluorescence spectra of the single tryptophan residues in HERA and smMLCK were acquired in the presence of saturating Ca^{2+} as the ratio of CaM/peptide was increased up to 3:1. At the conclusion of each titration, EDTA was added to demonstrate the Ca^{2+}dependence of binding. The peptide concentration was 10 mM and was held constant during the titrations. The excitation wavelength was 295 nm.

Subtasks c). Perform NMR experiments with either the phosphorylated or non-phosphorylated peptide bound to ERa to assess affinity and structural changes upon phosphorylation and analyze data (Months 15-22). See below

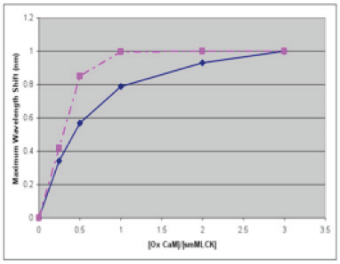
Subtasks d). Perform NMR experiments to monitor structural changes in ERa upon phosphorylation of Ser 305, with and without CaM bound and analyze data (Months 17-24). See below

Subtasks e). Perform SPR experiments on CaM bound to phosphorylated ERa with and without TAM bound, to determine the affinity change of phosphorylated ERa with CaM in the presence of TAM (Months 24-36). See below

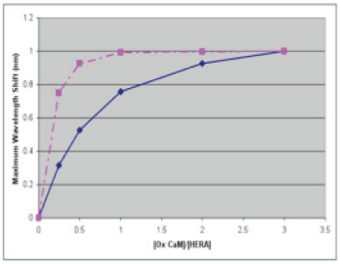
Subtasks c-e). In order to begin to address the overall aim of antiestrogen resistance and CaM oxidation, we need to determine if oxidized CaM (CaMox) still binds to (and activates) ERa. Towards this goal, initial experiments were performed using fluorescence spectroscopy to monitor the interaction between CaM, L9-CaM, and CaMox with the $HERA_{287-311}$ peptide. These results are shown in **Figure 22** and **Figure 23**.

The results in **Figure 22** and **Figure 23** indicate that oxidation of the methionine residues in CaM decreases the affinity of CaM for $HERA_{287-311}$, but not to a limited degree. The results suggest that CaMox most likely can still activate ERa, thus, oxidative stress most likely cannot eliminate activation of ERa by CaM. This is a key result, and suggests that blocking ERa activation by CaM with TAM (or its metabolites) can be reversed by oxidative stress, and therefore could be a component of oxidative stress induced antiestrogen resistance.

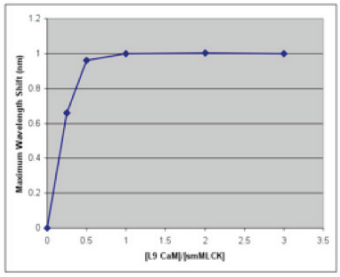
Finally, we have initiated fluorescence and NMR experiments with modified (phosphorylated, acetylated) peptides. The initial results suggest that none of these modifications produces a drastic affect on affinity for CaM. This in turn suggests that these



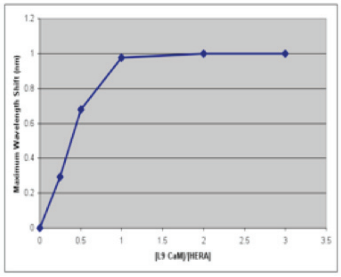
CaM/smMLCK
CaMox/smMLCK



CaM/HERA
CaMox/HERA



L9-CaM/smMLCK



L9-CaM/HERA

Fig. 23. Relative affinities of CaM, L9-CaM and CaMox for smMLCK and HERA. The maximum fluorescence emission wavelength shifts in the spectra in Figure Y were plotted as a function of the ratio of CaM species concentration to peptide concentration. The results indicate that oxidation of CaM reduces its affinity for smMLCK and HERA, but does not abrogate the interaction. L9-CaM also binds tightly to both smMLCK and HERA.

modifications are intended to provide fine or delicate control over CaM affinity and subsequently the ability of CaM to activate ERa. Because we chose to focus our efforts elsewhere during the past year, we have not been able to complete the SPR experiments proposed, and the NMR experiments have only been initiated. We will continue to pursue these experiments.

KEY RESEARCH ACCOMPLISHMENTS (cumulative):

- *Residues 286-552 of ERa, which includes the entire ligand binding domain and the putative calmodulin binding domain, does indeed bind calmodulin*. One of our goals, ultimately, is to determine the structural changes in the ligand binding domain of ERa that occur when calmodulin binds, and how these contribute to receptor activation. To date, we have not made as much progress towards this goal as we would have liked, but we have demonstrated that we can produce and purify this protein construct, and that it does bind to calmodulin (calmodulin affinity purification is the last step of the purification protocol). These studies are continuing with the goal of using this protein for NMR spectroscopy studies of the structural changes that occur in ERa when calmodulin binds and how these contribute to ERa activation.
- *The calmodulin binding region(s) of ERa reside(s) in amino acid residues 241-320*. Using an affinity tagged 241-320 (hinge region of ERa) construct (Trx-$ERA_{241-320}$), we demonstrated high affinity, calcium dependent binding to calmodulin.
- *A high affinity, calcium-dependent calmodulin binding site of ERa is further localized to residues 287-311*. Using a peptide synthesized by solid-phase methods ($HERA_{287-311}$), we have demonstrated a high affinity, calcium-dependent binding of calmodulin to residues 287-311 of ERa. The amino acid sequence represents a non-canonical calmodulin binding region, but nevertheless shares many of the properties of traditional high affinity calmodulin binding sites (basic, amphiphilic, probably helical).
- *A shorter region of ERa ($HERA_{295-311}$) does not represent the full calmodulin binding region*. An independent investigation by another research group has suggested that residues 295-311 of ERa constitute the calmodulin binding region. Our results indicate that this is incorrect. Firstly, our fluorescence results unequivocally demonstrate that W292 is buried in a hydrophobic pocket of one of the globular domains of calmodulin (this residue is not present in the shorter peptide). Secondly, we have compared directly the relative affinities of the longer and shorter peptides, and the shorter peptide clearly displays a much lower affinity for calmodulin.
- *An additional, low affinity calmodulin binding region was ruled out by additional control experiments.* Initial results suggested an additional, low-affinity, non-sequence specific CaM binding region in the linker region. Extensive controls ruled this out.
- *The high affinity calmodulin binding region (287-311) of ERa contains three residues subject to posttranslational modifications that can potentially mediate calmodulin affinity*. Residues S305, K302 and K303 of ERa have all been shown to be posttranslationally modified (the serine phosphorylated, the lysines acetylated). Calmodulin binds to basic (and amphiphilic) domains, so increasing the negative charge in this region (phosphorylating the serine) or decreasing the positive charge (acetylating the lysines) would be expected to decrease the affinity for calmodulin. Thus, it appears that nature has chosen to mediate ERa activation by attenuating calmodulin affinity by posttranslational modification.
- *The interaction of the ERa peptide ($HERA_{287-311}$) with calmodulin results in structural changes in calmodulin that suggest that the structural collapse of calmodulin around the bound peptides is less dramatic than with canonical CaM binding domains, that the complex of CaM with ERa is somewhat elongated, and that unusual structural changes are occurring in the C-terminal binding pocket*. Using NMR spectroscopy, we have determined how the chemical

shifts of calmodulin change when the ERa calmodulin binding peptide binds. The changes indicate that, compared to complexes of calmodulin with other high affinity targets, the complex of calmodulin with the ERa peptide is extended or elongated rather than collapsed. This result was confirmed by SAXS studies that showed clearly that the envelope of the complex was longer and less globular than that of the control complex (CaM with MLCK peptide bound). We also observed some large, notable and atypical chemical shift changes in the C-terminal globular domain of calmodulin suggesting some novel structural attributes. We are in the process of determining a high resolution structure of this complex using NMR spectroscopy.

- *The calmodulin binding region of ERa (HERA$_{287-311}$) adopts both helical structure and random coil structure when bound to calmodulin*. Most calmodulin binding regions of target proteins adopt (basic, amphiphilic) helical character when they bind to calmodulin. The ERα peptide is no exception. However, in contrast to canonical CaM binding regions, a significant population of extended structure is also adopted (random coil). In the absence of calmodulin, circular dichroism indicates a total lack of helical structure, but the peptide clearly adopts some helical structure when bound to calmodulin.
- *The ligand binding domain of ERa is amenable to NMR studies*. One important source of risk to the proposed studies was whether or not we would be able to acquire quality NMR data on the ligand binding domain of ERa (and the extended ligand binding domain that includes the CaM binding region). We have now demonstrated that we can do so, and without carboxymethylation of the cys residues, so that the system is more native like. This is a bid accomplishment, and one that will allow very important structural studies to continue
- *An isotopically labeled recombinant CaM binding region of ERa can be produced*. We were successful in producing a recombinant peptide corresponding to the CaM bindng region of ERa. This was an important accomplishment, as it paves the way for completion of the structure of the complex of CaM with the CaM binding region of ERa. This structure will provide critical information on the nature of the activation function promoted by CaM binding to ERa.
- *Calmodulin binds to raloxifene*. To our knowledge, this is the first demonstration of the fact that CaM can bind to antiestrogens used for breast cancer therapies other than tamoxifen and hydroxytamoxifen.
- *Oxidation of the methionine residues in calmodulin results in the inability of calmodulin to bind to tamoxifen, hydroxytamoxifen and raloxifene*. It has been conjectured that the beneficial consequences of tamoxifen therapy for estrogen dependent breast cancers results from the binding of tamoxifen to calmodulin, which inhibits the ability of calmodulin to activate the receptor. Under conditions of high oxidative stress in breast cancer tissues, if the methionine residues in calmodulin are oxidized, tamoxifen no longer binds to calmodulin. This could be one mechanism by which antiestrogen resistance could develop.
- *The inability of oxidized calmodulin to bind tamoxifen/hydroxytamoxifen/raloxifene is due to altered polarity of the tamoxifen binding sites on calmodulin*. Control experiments show that replacement of all methionine residues in calmodulin with leucine does not substantially alter tamoxifen binding. Therefore, the polarity changes induced by oxidation of the methionine residues in calmodulin to methionine sulfoxide most likely is the cause of the results observed.
- *The complex of CaM with hydroxytamoxifen is more amenable to NMR characterization than the CaM*-TAM complex. Apparently, the very poor aqueous solubility of tamoxifen results in the inability to completely saturate the TAM binding sites on CaM, resulting in some conformational averaging in the NMR spectra. However, hydroxytamoxifen is slightly more soluble, resulting in compelte saturation. Thus, the complex of CaM with OHTAM is somewhat more amenable to high resolution characterization by NMR than the CaM-TAM complex.
- *Oxidation of CaM does not abrogate binding to ERa*. Our results indicate that oxidation of the methionine residues in CaM decreases the affinity of CaM for HERA$_{287-311}$, but not to a limited degree. The results suggest that CaMox most likely can still activate ERa, thus, oxidative

stress most likely cannot eliminate activation of ERa by CaM. This is a key result, and suggests that blocking ERa activation by CaM with TAM (or its metabolites) can be reversed by oxidative stress, and therefore could be a component of oxidative stress induced antiestrogen resistance.

- *Posttranslational modifications in the CaM binding region of ERa provide for delicate control of ERa activation by CaM*. The initial results of studies assessing the affinity of CaM for peptides corresponding to the CaM binding region of ERa that have been modified based on known posttranslational modifications of this region indicate that none of these modifications produces a drastic affect on affinity for CaM. This in turn suggests that these modifications are intended to provide fine or delicate control over CaM affinity and subsequently the ability of CaM to activate ERa.

REPORTABLE OUTCOMES:

Publications (cumulative):

We are completing some of the studies and will submit the results for publication in first-tier journals. We anticipate at least three publications, if not more, will result.

Abstracts/Presentations (cumulative):

Including a presentation scheduled for early 2011, we have presented the results of this work at eight local/regional or national/international scientific conferences. Full abstracts for these appear in the "APPENDICES" section.

Urbauer, J. L., Jolly, C., Jones, B., Henderson, E., and Bieber Urbauer, R. J. (2011) Structural Studies of Calmodulin Activation of Estrogen Receptor Alpha. 55th Annual Meeting of the Biophysical Society, March 5-9, Baltimore, MD.

Urbauer, J. L., Jolly, C. Johnson, S., Galdo, J., Cross, M., Elliott, M., Nooromid, M., Cho, L., Cheung, N., Jones, B., Henderson, E. and Bieber Urbauer, R., (2010) Calmodulin Activation of Estrogen Receptor Alpha. 24th Annual Symposium of the Protein Society, August 1-5, San Diego, CA.

Urbauer, J. L., Jolly, C. Johnson, S., Galdo, J., Cross, M., Elliott, M., Nooromid, M., Cho, L., Cheung, N., and Bieber Urbauer, R., (2010) Activation of Estrogen Receptor Alpha by Calmodulin. 54th Annual Meeting of the Biophysical Society, February 20-24, San Francisco, CA.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Nooromid, M., and Urbauer, J. L. (2009) Estrogen Receptor Alpha Activation by Calmodulin. The Georgia Cancer Coalition 2009 Research Symposium, November 5-6, Athens, GA.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Nooromid, M., and Urbauer, J. L. (2009) Estrogen Receptor Alpha Activation by Calmodulin. 23rd Annual Symposium of the Protein Society, July 25-29, Boston, MA.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Elliott, M. C., Nooromid, M. and Urbauer, J. L. (2008) Calmodulin mediated estrogen receptor alpha activation and antiestrogen resistance. 22nd Annual Symposium of the Protein Society, July 19-23, San Diego, CA.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., and Urbauer, J. L. (2008) Mechanistic Basis of Calmodulin Mediated Estrogen Receptor Alpha Activation and Antiestrogen Resistance. Era of Hope 2008 Meeting, June 25-28, Baltimore, MD.

Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Bieber Urbauer, R. J., and Urbauer, J. L. (2007) The Interaction of Calmodulin with Estrogen Receptor Alpha. Southeast Regional Meeting of the American Chemical Society, October 24-27, Greenville, SC.

Salaried personnel (cumulative):

The following individuals received pay for working on this project:
Jeffrey Urbauer (PI), Ramona J. Bieber Urbauer (Senior Scientist), Carrie Jolly (postdoctoral researcher), Leah Cho (technical assistance), Marie Cross (technical assistance), Nathan Olive (technical assistance), Jane Ullah (technical assistance), Madeline Elliott (technical assistance), Alicia Dery (technical assistance), Erik Henderson (graduate student / technical assistance).

Education/Training/Employment/Research Opportunities (cumulative):

Undergraduate training. To date, nine undergraduate students, from both UGA, neighboring institutions, and institutions outside of the Southeast, have participated in, or are currently participating in, this project in my laboratory and have received important scientific training both in basic protein biochemistry and in cancer biology. These students (listed below) are all pursuing professional careers in science and medically related fields.

I'll also note that five of these students are co-authors of at least one of the presentations listed above and most, if not all, will be co-authors on planned journal submissions.

-Madeline Elliott (Honors Program, UGA), currently attending Mercer University School of Medicine
-Michael Nooromid (Honors Program, UGA), currently attending New York University School of Medicine
-John Galdo (UGA), currently in pharmacy school at The University of Georgia
-Marie Cross (UGA), currently attending College of Dental Medicine, The Medical University of South Carolina
-Aisha Mahmood (UGA), undergraduate Honors Student, The University of Georgia
-Ashley Itua (UGA), undergraduate Biochemistry and Molecular Biology major, currently pursuing admittance to medical school
-*Noelle Cheung (Carnegie Mellon University), graduate school or medical school
-*Leah Cho (Denver University), graduate school or medical school
-**Savannah Johnson (Piedmont College), attending graduate school, Emory University Department of Chemistry

*Noelle and Leah were undergraduate participants in the SURO (Summer Undergraduate Research Opportunity) program in the Chemistry Department at UGA (summer, 2008).
**Savannah was a participant in the SURO (Summer Undergraduate Research Opportunities) program in the Department of Chemistry at UGA during the summer of 2007.

Graduate Training. Currently, a graduate student, Erik Henderson, is performing his graduate thesis research in the Urbauer lab and is working on this project. Erik is a graduate student in the Department of Biochemistry and Molecular Biology at The University of Georgia.

A new graduate student, Henry Niedermaier, from the Department of Chemistry, is just

now joining our research group and will be working on this project pursuing the proposed NMR studies.

Postdoctoral Training. Likewise, a Postdoctoral Researcher in my laboratory, Dr. Carrie Jolly, worked on various aspects of this project during her 20 month stay. Dr. Jolly has since moved on to Montana with her family, where she is now employed as a postdoctoral researcher at the National Institutes of Health, National Institute of Allergy and Infectious Diseases, at the Rocky Mountain Laboratories.

Technical Training. A number of individuals have worked on this project in a technical capacity (Leah Cho, Nathan Olive, Jane Ullah, Alicia Dery) for limited times, contributing to their experience and abilities as scientists and professionals.

High School Student Training. Finally, this summer a high school student, Mr. Minhyuk (Michael) Song, from a high school in a neighboring community (Brookwood High School, Snellville, GA), is pursuing a summer research opportunity in the Urbauer laboratory, working with a graduate student (**Erik Henderson**) on this project. Michael is part of the "Young Dawgs" summer research program. His father is a professor in the Department of Physics at UGA.

CONCLUSION and "SO-WHAT" section (cumulative):

Knowledge of the precise binding site or sites for CaM on ERa is essential ultimately for understanding, from a structural and mechanistic perspective, how calmodulin binding to ERa activates ERa. Because it is apparent that the C-terminal end of the CaM binding domain is part of the ligand binding domain of ERa, CaM binding most likely affects the structure of the ligand binding domain, and therefore the current mechanisms for E2 activation and antiestrogen inactivation are at least incomplete if not incorrect. Furthermore, recent studies have "localized" the CaM binding domain to a short section of the hinge region, and we have demonstrated that this is incorrect. In addition, we have found what appears to be a second, lower affinity CaM binding site in the N-terminal region of the hinge, which appears to be Ca^{2+}-independent. Allthough this site apparently is non-specific (not sequence specific), this could have important implications for CaM binding and ERa activation, and could represent a mechanism to improve affinity of CaM for ERa without the need for sequence specificity.

CaM binding domains of dozens of proteins activated by CaM are most always basic, amphiphilic, and adopt helical structure when bound to CaM. The high affinity site that we have localized on ERa appears similar in these respects, except that only part of the binding region adopts helical structure, and the remainder apparently does not (random coil). The more rigid helical structure often adopted by these domains is integral to the mechanism of activation, and often represents the first, obligatory step in the activation process. In this case, perhaps the activation process only necessitates partial helical character. Thus, it is critical to understand these structural changes in order to define mechanistically how activation occurs. In the same vein, the structural changes that occur in CaM when it binds to these domains is also important. These changes may be very large, as is the case for binding to the MLCK (discussed above), or smaller, as is the case for ERa. This is important, as it signifies that a large scale "collapse" of CaM around the high affinity domain in ERa is less dramatic, and reflects fundamantal structural attributes associated with the activation process, In the case of ERa, we suspect that these more modest structural changes result from the fact that CaM can bind to two CaM binding domains of ERa simultaneously, and that the crowding resulting from the presence of two peptides does not permit a dramatic collapse, as is observed for MLCK (for instance).

Our hypothesis is that CaM and oxidative stress are integral to the process of antiestrogen development. Our rationale is as follows. It has been suggested that tamoxifen (TAM) binding to CaM blocks CaM activation of ERa, and therefore contributes positively to tamoxifen therapy for estrogen dependent breast cancer. The oxidative stress level (and levels of reactive oxygen species) in breast cancer tissue is high, and because the methionine residues in CaM are easily oxidized, we tested whether oxidized CaM could still bind TAM. It does not. Furthermore, hydroxytamoxifen (one of the active metabolites of TAM) and raloxifene (another antiestrogen, but chemically distince from TAM and metabolites) display the same behavior. If oxidized CaM can still bind and activate ERa, then TAM/OHTAM/raloxifene would not be able to as effectively inhibit CaM activation of ERa when CaM is oxidized. Thus, this would be a mechanism for development of antiestrogen resistance. Furthermore, we know also know that CaM oxidation does not eliminate binding to ERa, indicating that CaMox most likely can still activate ERa, thus, oxidative stress most likely cannot eliminate activation of ERa by CaM. This is a key result, and suggests that blocking ERa activation by CaM with TAM (or its metabolites) can be reversed by oxidative stress, and therefore could be a component of oxidative stress induced antiestrogen resistance.

Overall, our studies to date have been very successful and informative. We have established a very firm foundation for continuing the work that we originally proposed. We anticipate that the investment by the CDMRP and Breast Cancer Research Program will continue to see returns.

REFERENCES:

1. Gangloff, M., Ruff, M., Eiler, S., Duclaud, S., Wurtz, J. M., and Moras, D. (2001) Crystal structure of a mutant hERalpha ligand-binding domain reveals key structural features for the mechanism of partial agonism, *J Biol Chem 276*, 15059-15065.
2. Schwabe, J. W., Chapman, L., Finch, J. T., and Rhodes, D. (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements, *Cell 75*, 567-578.
3. Hoeflich, K. P., and Ikura, M. (2002) Calmodulin in action: diversity in target recognition and activation mechanisms, *Cell 108*, 739-742.
4. Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) Calmodulin target database, *J Struct Funct Genomics 1*, 8-14.
5. Gallo, D., Jacquemotte, F., Cleeren, A., Laios, I., Hadiy, S., Rowlands, M. G., Caille, O., Nonclercq, D., Laurent, G., Jacquot, Y., and Leclercq, G. (2007) Calmodulin-independent, agonistic properties of a peptide containing the calmodulin binding site of estrogen receptor alpha, *Mol Cell Endocrinol 268*, 37-49.
6. Gallo, D., Jacquot, Y., Laurent, G., and Leclercq, G. (2008) Calmodulin, a regulatory partner of the estrogen receptor alpha in breast cancer cells, *Mol Cell Endocrinol 291*, 20-26.
7. Ikura, M., Kay, L. E., Krinks, M., and Bax, A. (1991) Triple-resonance multidimensional NMR study of calmodulin complexed with the binding domain of skeletal muscle myosin light-chain kinase: indication of a conformational change in the central helix, *Biochemistry 30*, 5498-5504.
8. Robertus, C., Philipse, W. H., Joosten, J. G. H., and Levine, Y. K. (1989) Solution of the Percus-Yevick Approximation of the Multicomponent Adhesive Spheres System Applied to the Small-Angle X-Ray-Scattering from Microemulsions, *Journal of Chemical Physics 90*, 4482-4490.
9. Svergun, D., and Feigin, L. A., (Eds.) (1989) *Structure Analysis by Small-Angle X-ray and Neutron Scattering*, Plenum Press, Sec. Ed. New York.
10. Svergun, D. I., Konarev, P. V., Volkov, V. V., Koch, M. H. J., Sager, W. F. C., Smeets, J., and Blokhuis, E. M. (2000) A small angle x-ray scattering study of the droplet-cylinder transition in oil-rich sodium bis(2-ethylhexyl) sulfosuccinate microemulsions, *Journal of Chemical Physics 113*, 1651-1665.

11. Franke, D., and Svergun, D. I. (2009) DAMMIF, a program for rapid ab-initio shape determination in small-angle scattering, *Journal of Applied Crystallography 42*, 342-346.
12. Jin, K. S., Park, J. K., Yoon, J., Rho, Y., Kim, J. H., Kim, E. E., and Ree, M. (2008) Small-angle X-ray scattering studies on structures of an estrogen-related receptor alpha ligand binding domain and its complexes with ligands and coactivators, *Journal of Physical Chemistry B 112*, 9603-9612.
13. Michalides, R., Griekspoor, A., Balkenende, A., Verwoerd, D., Janssen, L., Jalink, K., Floore, A., Velds, A., van't Veer, L., and Neefjes, J. (2004) Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer, *Cancer Cell 5*, 597-605.
14. Lim, Y. C., Desta, Z., Flockhart, D. A., and Skaar, T. C. (2005) Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen, *Cancer Chemother Pharmacol 55*, 471-478.
15. Lim, Y. C., Li, L., Desta, Z., Zhao, Q., Rae, J. M., Flockhart, D. A., and Skaar, T. C. (2006) Endoxifen, a secondary metabolite of tamoxifen, and 4-OH-tamoxifen induce similar changes in global gene expression patterns in MCF-7 breast cancer cells, *J Pharmacol Exp Ther 318*, 503-512.
16. Wu, X., Hawse, J. R., Subramaniam, M., Goetz, M. P., Ingle, J. N., and Spelsberg, T. C. (2009) The tamoxifen metabolite, endoxifen, is a potent antiestrogen that targets estrogen receptor alpha for degradation in breast cancer cells, *Cancer Res 69*, 1722-1727.
17. Bartlett, R. K., Bieber Urbauer, R. J., Anbanandam, A., Smallwood, H. S., Urbauer, J. L., and Squier, T. C. (2003) Oxidation of Met144 and Met145 in calmodulin blocks calmodulin dependent activation of the plasma membrane Ca-ATPase, *Biochemistry 42*, 3231-3238.

APPENDICES:

Abstracts (cumulative):

Urbauer, J. L., Jolly, C., Jones, B., Henderson, E., and Bieber Urbauer, R. J. (2011) Structural Studies of Calmodulin Activation of Estrogen Receptor Alpha. 55th Annual Meeting of the Biophysical Society, March 5-9, Baltimore, MD.

The goal is to determine the mechanism of calcium-dependent activation of estrogen receptor alpha (ERa) by calmodulin (CaM) and to ascertain how oxidative stress and oxidative modifications mediate the interactions between CaM, ERa and antiestrogens. Systemic endocrine/antiestrogen therapy is among the most common treatments for estrogen-dependent breast cancers. ERa is the primary target for antiestrogen therapies, and antiestrogen drugs such as tamoxifen and its metabolites (4-hydroxytamoxifen, endoxifen) bind tightly to ERa and inhibit its ability to activate transcription. Recently, it was demonstrated that CaM is an obligate ERa activator. Interestingly, antiestrogens that bind tightly to ERa also bind tightly to CaM. It has been suggested that therapeutic benefit of antiestrogens for estrogen-dependent breast cancers may derive partially from CaM antagonism. Towards our goal of understanding how CaM activates ERa, we have localized the CaM binding region of ERa and initiated structural studies to determine the structure of the complex of CaM with the ERa CaM binding region. Using NMR and SAXS we find that CaM bound to ERa is somewhat extended structurally compared to high affinity CaM complexes. Circular dichroism and fluorescence studies indicate high affinity between CaM and the CaM binding domain of ERa and that upon binding to CaM the CaM binding region of ERa adopts only partial helical character. Binding of CaM to hydrophobic antiestrogens (tamoxifen, 4-hydroxytamoxifen, endoxifen, raloxifene) is eliminated when methionine residues in CaM are oxidized. However, oxidation does not eliminate binding to ERa. Control experiments with CaM mutants (leucine for methionine) indicate methionine residues are not essential for antiestrogen binding. The results are important for understanding CaM activation of ERa and the link between oxidative stress and antiestrogen resistance development. (Supported by the DOD, the Georgia Cancer Coalition, Bruker AXS and the University of Georgia).

Urbauer, J. L., Jolly, C. Johnson, S., Galdo, J., Cross, M., Elliott, M., Nooromid, M., Cho, L., Cheung, N., Jones, B., Henderson, E. and Bieber Urbauer, R., (2010) Calmodulin Activation of Estrogen Receptor Alpha. 24th Annual Symposium of the Protein Society, August 1-5, San Diego, CA.

The alpha isoform of the estrogen receptor (ERa) is the principal target for systemic endocrine/antiestrogen therapy for estrogen-dependent breast cancers. It has been known for some time that ERa is subject to calcium-dependent activation by calmodulin (CaM), and recently this interaction with CaM was demonstrated to be essential for activation. CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM), and its metabolites. It has been suggested that therapeutic benefit of antiestrogens for estrogen-dependent breast cancers may derive partially from CaM antagonism. We are interested in defining the molecular mechanism of CaM activation of ERa and establishing how oxidative stress might mediate the interactions of CaM with ERa and antiestrogens. Recently we localized the CaM binding region of ERa and initiated NMR studies to determine the solution structure of the complex of CaM with the ERa CaM binding region. Chemical shift changes indicate that CaM bound to ERa is more extended structurally compared to typical CaM complexes and identify important structural changes in the

C-terminal binding pocket of CaM. The ERa CaM binding region adopts partial helical character upon CaM binding. Oxidation of the methionine residues in CaM eliminates binding to TAM, its metabolites and related hydrophobic antiestrogens, but these still bind to mutant CaM where all methionine residues are replaced by leucine. Oxidation reduces the affinity of CaM for ERa but does not eliminate it. Methionine oxidation results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results are important for a comprehensive understanding of CaM activation of ERa and the link between oxidative stress and development of antiestrogen resistance.

Urbauer, J. L., Jolly, C. Johnson, S., Galdo, J., Cross, M., Elliott, M., Nooromid, M., Cho, L., Cheung, N., and Bieber Urbauer, R., (2010) Activation of Estrogen Receptor Alpha by Calmodulin. 54th Annual Meeting of the Biophysical Society, February 20-24, San Francisco, CA.

The alpha isoform of the estrogen receptor (ERa) is the principal target for systemic endocrine/antiestrogen therapy for estrogen-dependent breast cancers. ERa is subject to calcium-dependent activation by calmodulin (CaM), and recently this interaction with CaM was demonstrated to be essential for activation. CaM also binds tightly to antiestrogens, including the widely used chemotherapeutic tamoxifen (TAM) and its metabolites. It has been suggested that therapeutic benefit of antiestrogens for estrogen-dependent breast cancers may derive partially from CaM antagonism. We are interested in defining the molecular mechanism of CaM activation of ERa and establishing how oxidative stress might mediate the interactions of CaM with ERa and antiestrogens. Recently we localized the CaM binding region of ERa and initiated NMR studies to determine the solution structure of the complex of CaM with the ERa CaM binding region. Chemical shift changes indicate that CaM bound to ERa is more extended structurally compared to typical CaM complexes and identify important structural changes in the C-terminal binding pocket of CaM. The ERa CaM binding region adopts partial helical character upon CaM binding. Oxidation of the methionine residues in CaM eliminates binding to TAM, its metabolites and related hydrophobic antiestrogens, but these still bind to mutant CaM where all methionine residues are replaced by leucine. Oxidation reduces the affinity of CaM for ERa but does not eliminate it. Methionine oxidation results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results are important for a comprehensive understanding of CaM activation of ERa and the link between oxidative stress and development of antiestrogen resistance.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Nooromid, M., and Urbauer, J. L. (2009) Estrogen Receptor Alpha Activation by Calmodulin. The Georgia Cancer Coalition 2009 Research Symposium, November 5-6, Athens, GA.

The alpha isoform of the estrogen receptor (ERa) is the principal target for systemic endocrine/antiestrogen therapy for estrogen-dependent breast cancers. It is known that ERa is subject to calcium-dependent activation by calmodulin (CaM), and recently this interaction was demonstrated to be essential for activation. CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM), and its metabolites. It has been suggested that therapeutic benefit of antiestrogens for estrogen-dependent breast cancers may derive partially from CaM antagonism.

We are interested in defining the molecular mechanism of CaM activation of ERa and establishing how oxidative stress might mediate the interactions of CaM with ERa and antiestrogens. We have localized the CaM binding region of ERa and initiated NMR studies to

determine the solution structure of the complex of CaM with the ERa CaM binding region. Results indicate that CaM bound to ERa is more extended structurally than typical CaM complexes and the ERa CaM binding region adopts partial helical character upon CaM binding. Oxidation of the methionine residues in CaM eliminates binding to TAM, its metabolites and related hydrophobic antiestrogens, but these still bind to mutant CaM where all methionine residues are replaced by leucine. Oxidation reduces the affinity of CaM for ERa but does not eliminate it. Methionine oxidation results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results are important for a comprehensive understanding of CaM activation of ERa and the link between oxidative stress and development of antiestrogen resistance.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Nooromid, M., and Urbauer, J. L. (2009) Estrogen Receptor Alpha Activation by Calmodulin. 23rd Annual Symposium of the Protein Society, July 25-29, Boston, MA.

The alpha isoform of the estrogen receptor (ERα) is the principal target for systemic endocrine/antiestrogen therapy for estrogen-dependent breast cancers. It has been known for some time that ERα is subject to calcium-dependent activation by calmodulin (CaM), and recently this interaction with CaM was demonstrated to be essential for activation. CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM), and its metabolites. It has been suggested that therapeutic benefit of antiestrogens for estrogen-dependent breast cancers may derive partially from CaM antagonism. We are interested in defining the molecular mechanism of CaM activation of ERα and establishing how oxidative stress might mediate the interactions of CaM with ERα and antiestrogens. Recently we localized the CaM binding region of ERα and initiated NMR studies to determine the solution structure of the complex of CaM with the ERα CaM binding region. Chemical shift changes indicate that CaM bound to ERα is more extended structurally compared to typical CaM complexes and identify important structural changes in the C-terminal binding pocket of CaM. The ERα CaM binding region adopts partial helical character upon CaM binding. Oxidation of the methionine residues in CaM eliminates binding to TAM, its metabolites and related hydrophobic antiestrogens, but these still bind to mutant CaM where all methionine residues are replaced by leucine. Oxidation reduces the affinity of CaM for ERα but does not eliminate it. Methionine oxidation results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results are important for a comprehensive understanding of CaM activation of ERα and the link between oxidative stress and development of antiestrogen resistance.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Elliott, M. C., Nooromid, M. and Urbauer, J. L. (2008) Calmodulin mediated estrogen receptor alpha activation and antiestrogen resistance. 22nd Annual Symposium of the Protein Society, July 19-23, San Diego, CA.

Estrogens and estrogen receptor alpha (ERa) are central to estrogen-dependent breast cell carcinoma induction and proliferation. ERa is the principal target for systemic endocrine/antiestrogen therapy, underscoring its biological relevance and medical importance. Recently, it was established that calcium-dependent activation by calmodulin (CaM) is essential for estrogen-dependent ERa activity and that the active species is the CaM-ERa complex. CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM). The therapeutic effects of antiestrogens are indicated to be due, in part, to CaM antagonism. Our objectives include establishing the molecular mechanism whereby CaM activates estradiol-dependent ERa transcription and

defining the role of oxidative stress in mediating CaM-ERa and CaM-antiestrogen interactions. We have localized the CaM binding region of ERa to a 25 amino acid segment in the ERa hinge region and have initiated NMR spectroscopy studies to determine the structure of the complex of CaM with this CaM binding region. Based on chemical shift changes, the collapse of CaM around the ERa CaM binding domain is much less dramatic than observed for complexes of CaM with prototypical binding domains, with relatively large structural changes occur in the C-terminal domain of CaM. These results suggest that CaM bound to ERa is more extended structurally compared to typical CaM complexes and signify important structural changes in the C-terminal binding pocket of CaM. Oxidation of the methionine residues in CaM eliminates binding to TAM and hydroxy-TAM. TAM binding to mutant CaM where all methionine residues are replaced by leucine is unaffected by the leucine substitutions. Methionine oxidation results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results are important for a comprehensive understanding of CaM activation of ERa and the link between oxidative stress and development of antiestrogen resistance.

Bieber Urbauer, R. J., Jolly, C. E., Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., and Urbauer, J. L. (2008) Mechanistic Basis of Calmodulin Mediated Estrogen Receptor Alpha Activation and Antiestrogen Resistance. Era of Hope 2008 Meeting, June 25-28, Baltimore, MD.

Estrogens and estrogen receptor alpha (ERa) are central to estrogen-dependent breast cell carcinoma induction and proliferation. ERa is the principal target for systemic endocrine/antiestrogen therapy, underscoring its biological relevance and medical importance. Recently it has been established that calcium-dependent activation by calmodulin (CaM) is essential for estrogen-dependent ERa activity, and that the active species is the CaM-ERa complex (Li, L., Li, Z., and Sacks, D. B. (2005) *J Biol Chem 280*, 13097-104.). CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM). The therapeutic effects of antiestrogens, like those of other CaM antagonists, are indicated to be due, in part, to the direct interaction with CaM.

Oxidative stress – estrogen-induced oxidative stress and constitutive oxidative stress – is indicated in estrogen-dependent breast cancer tissues. Oxidative stress is also implicated as mediating development of resistance of breast cancers to antiestrogens. TAM is also implicated in inducing a potent oxidative stress response in breast cancer tissue. It has been demonstrated in other oxidatively stressed tissues (senescent brain), that increased levels of reactive oxygen species and the failure of cellular repair mechanisms conspire to cause accumulation of oxidized CaM species (where one or more of the nine methionine residues are oxidized to the sulfoxides), altering intracellular calcium homeostasis. Oxididation of CaM can reduce its ability to activate some target proteins, without necessarily reducing binding affinity. Because there are nine methionine residues in CaM, most of which interact with the CaM binding domains of target proteins, the effects of oxidation can be specific for particular methionine residues.

Our objectives include establishing the molecular mechanism, including the structural details, by which CaM activates estradiol-dependent ERa transcription, and defining the role of oxidative stress in mediating CaM-ERa and CaM-antiestrogen interactions. Towards these goals we have localized the CaM binding region of ERa to a 25 amino acid segment in the hinge region of ERa. We have initiated studies using NMR spectroscopy to determine the structure of the complex of CaM with this CaM binding region of ERa. To date, based on chemical shift changes, we have found the collapse of CaM around the ERa CaM binding domain is much less dramatic than observed for complexes of CaM with prototypical binding domains, and that relatively large structural changes occur in the C-terminal domain of CaM. Oxidation of the methionine residues in CaM eliminates binding to TAM and hydroxy-TAM. TAM binding to CaM with all methionine

residues replaced by leucine is unaffected by the leucine substitutions. These results suggest that CaM bound to ERa is more extended structurally compared to typical CaM complexes and signify important structural changes in the C-terminal binding pocket of CaM. Oxidation of methionine residues in CaM results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results will be important for a comprehensive understanding of the principles governing CaM activation of ERa and the link between oxidative stress and development of antiestrogen resistance, in order to aid in the design and development of a new pharmaceuticals to treat breast cancers.

Johnson, S. J., Galdo, J. A., Cross, M. E., Elliott, M. C., Bieber Urbauer, R. J., and Urbauer, J. L. (2007) The Interaction of Calmodulin with Estrogen Receptor Alpha. Southeast Regional Meeting of the American Chemical Society, October 24-27, Greenville, SC.

Estrogen dependent breast cancers require the transcriptional activation activity of the estrogen receptor alpha (ERa). These account for approximately 70% of all breast cancers. In response to estrogen binding, a set of genes is activated by ERa. This facilitates growth and propagation of the cancer cells. The important calcium-binding signaling protein calmodulin (CaM) binds ERa, and, recently, it was demonstrated that CaM is essential for activation of ERa transcriptional activity. Here we present progress towards defining the CaM binding region of ERa and the structural changes in CaM upon interaction with ERa, and towards understanding potential cellular mechanisms for mediating activation of ERa by CaM. As a first step towards elucidating the mechanism of ERa activation by CaM, we have produced a segment of the ERa protein (residues 241-320) as a thioredoxin fusion and demonstrated binding to CaM. A short, 25 amino acid section of this segment of ERa, suspected to comprise the CaM binding sequence, was produced, as were three derivatives containing modifications known to occur naturally. Fluorescence titration experiments demonstrated that these peptides all bind to CaM, and they all bind with rare 2:1 (peptide:CaM) stoichiometries. Their relative binding affinities are consistent with established principles for CaM interactions with target domains. The complex of CaM with the wild-type peptide was studied using NMR spectroscopy. The chemical shifts of CaM were assigned using triple resonance methods. The changes in chemical shifts upon peptide binding suggest an altered binding mode relative to typical complexes of CaM with target peptides.

SUPPORTING DATA:

All figures are embedded in the text (above), along with their respective figure legends